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FEBRUARY, 1986

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $20.00. Subscription per volume (three issues), $50.00 ($100.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor, or Pamela Clapp, Assistant Editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

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ISSN 0006-3185

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The BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

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THE BIOLOGICAL BULLETIN (ISSN 0006-3185)

POSTMASTER: Send address changes to THE BIOLOGICAL BULLETIN,
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LANCASTER PRESS, INC., LANCASTER, PA

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Reference: *Biol. Bull.* **170:** 1–10. (February, 1986)

CHEMICAL STIMULANTS FOR A COMPONENT OF FEEDING BEHAVIOR IN THE COMMON GULF-WEED SHRIMP *LEANDER TENUICORNIS* (SAY)

BRUCE R. JOHNSON[1] AND JELLE ATEMA

Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT

In a shipboard study we examined the chemical feeding cues for a component of feeding behavior in the common gulf-weed shrimp, *Leander tenuicornis,* a dominant crustacean within the pelagic *Sargassum* community. We used the frequency of flexion of the chelae on the second legs to the mouth or maxillipeds as an assay for chemosensitivity. Of 28 single compounds tested, taurine was the most stimulatory followed by beta-alanine, glutathione, trimethylamine, and succinic acid. Proline inhibited spontaneous flexion of the chelae. Taurine was the only single stimulant within a mixture of 15 compounds and it alone could account for the mixture's stimulatory ability. Taurine is also an important feeding and chemoreceptor stimulant for coastal crustaceans. It is present in high concentrations in crustacean prey but is maintained at low concentrations in background seawater. This combination of low background noise and reliably high stimulus intensity in food gives taurine excellent (feeding) signal properties in both coastal and pelagic environments.

INTRODUCTION

The pelagic *Sargassum* community is a unique assemblage of organisms which has attracted the attention of explorers and biologists for centuries. The first description of *Sargassum* fauna appeared in the diary of Christopher Columbus. On his initial voyage across the Atlantic, Columbus discovered *Sargassum* rafts and described crabs living within them, probably the Columbus crab *Planes minutus* (Chace, 1951). Most of the invertebrate fauna living on *Sargassum* rafts appear to have derived from littoral benthic populations and thus are characterized by Hedgepeth (1957) as a "displaced benthos" in the open ocean. Surveys of the invertebrate *Sargassum* fauna are found in Hentshel (1922), Timmerman (1932), Prat (1935), Weis (1968), Fine (1970), Butler *et al.* (1983), and Stoner and Greening (1984). Within this community there are approximately 100 species; the major groups include hydroids, anthozoans, turbellarians, polychaetes, gastropods, nudibranchs, bryozoans, pycnogonids, isopods, amphipods, copepods, and decapod crustaceans (Butler *et al.,* 1983).

Invertebrates of this pelagic community, as well as their close relatives living in littoral habitats, detect chemical stimuli against chemical noise levels in the background (Atema, 1985). For example, feeding behaviors and chemoreceptor responses of littoral crustaceans are stimulated by low molecular weight compounds such as amino acids and structurally related chemicals (Ache, 1982; Atema, 1985). These compounds must

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Received 8 October 1985; accepted 25 November 1985.

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be perceived against seawater background concentrations of total dissolved amino acids in the high nanomolar range (Daumas, 1976; Mopper and Lindroth, 1982). Only a few studies of crustacean chemoreception have examined chemical feeding cues in pelagic crustaceans (Fuzessery and Childress, 1976; Poulet and Ouellet, 1982; Hamner and Hamner, 1977; Hamner *et al.*, 1983) and none have seriously discussed chemical stimuli in relation to background concentration levels. A comparison of the chemical cues for feeding behaviors between crustaceans living on *Sargassum* and their littoral relatives may clarify the role that environmental constraints such as background noise levels play in determining the adequate stimuli for chemoreceptors.

The purpose of this investigation was to provide an initial description of the chemical feeding cues of a dominant crustacean of the *Sargassum* community, the common gulf-weed shrimp, *Leander tenuicornis* (Fig. 1). This study is the first to investigate chemical feeding cues in this community. The results are discussed in terms of signal detection.

MATERIALS AND METHODS

Collection and maintenance of animals

All experiments were carried out aboard the RV *Oceanus* (Cruise #165). Common gulf-weed shrimp were collected from *Sargassum* rafts dip-netted aboard ship at two locations off the mid-eastern coast of the United States (34°09′N, 76°03′W and 36°05′N, 74°16′W, approximately 50 miles east of Cape Lookout and Cape Hatteras respectively, along the western edge of the Gulf Stream). The animals were maintained in plastic bowls filled with ambient seawater changed 2–3 times daily. Ambient seawater (temperature range 12–23°C) was pumped into the shipboard lab through a clean garden hose from a seawater intake on the ship's bow. Two separate groups of animals were used in these experiments, one from each collection site. Fifteen animals were collected at the first site and tested for four days. Twenty animals were collected at the second site and tested for three days. All animals were tested individually and sequentially.

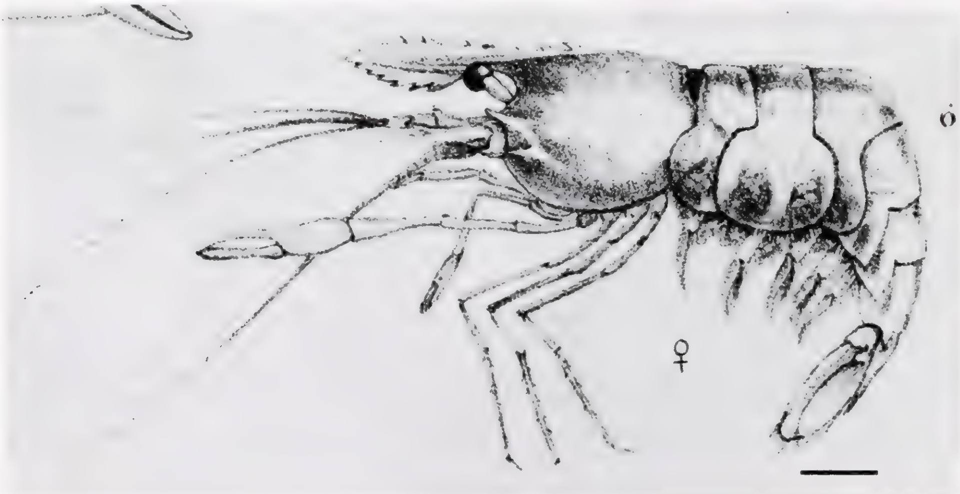

FIGURE 1. A female *Leander tenuicornis*, the common gulf-weed shrimp. Note the elongate slender chelae on the second legs. The frequency of movement of a chela to the mouth or to the maxillipeds was used as an assay to determine the shrimp's sensitivity to the chemicals in Table I. Drawing reproduced from a plate in Bate (1888). Bar equals 2 mm.

At the end of the experiments all animals were fixed in a solution of 10% formalin in seawater for later measurement and sex determination. Animals were not fed during the experimental time. This procedure avoided the development of any behavioral preference for particular odors (tastes) other than those associated with the normal feeding behavior on the *Sargassum*. Such shifts in behavioral odor responses due to recent feeding experience have been previously demonstrated in at least one decapod crustacean (Derby and Atema, 1981).

Stimulus preparation

Test stimuli are listed in Table I. The test stimuli were chosen from those tested previously as feeding or chemoreceptor stimulants in other crustaceans (Ache, 1982). All compounds were pre-weighed in amounts appropriate to prepare a 10^{-2} M solution, or for glycogen a 4 g/l solution, in 1 l of ambient seawater (SW). The preweighed compounds were stored either at 4°C or frozen. Test solutions were prepared on the day of an experiment and the pH adjusted with NaOH when necessary to that of SW (7.8). The only two test stimuli not prepared in this way were the SS, a mixture of 15 compounds (Table I), each at 10^{-2} M (total mixture molarity 1.5×10^{-1} M), used in electrophysiological studies of lobster smell and taste (Johnson and Atema, 1983;

TABLE I

Test stimuli

Compounds	Abbreviations
L-Alanine	Ala
L-Arginine	Arg
L-Aspartate	Asp
Betaine	Bet
Ethanol	EtOH
L-Glutamine	Gln
L-Glutamate	Glu
Glycine	Gly
Hydroxy-L-Proline	Hyp
L-Leucine	Leu
L-Lysine	Lys
Ammonium Chloride	NH_4
L-Proline	Pro
Sucrose	Suc
Taurine	Tau
Search Stimulus: a mixture of the above compounds	SS
Search Stimulus without Taurine	SS-Tau
Adenosine	Ade
Beta-Alanine	B-Ala
Citric Acid	Cit
Gamma-Amino-Butyric Acid	GABA
Glucose	Gluc
Glycogen	Glyc
Glutathione (reduced)	GSH
Homarine	Hom
Iso-L-Leucine	Ile
L-Methionine	Met
Succinic Acid	Succ
Sea Water	SW
Trimethylamine	TMA

Johnson *et al.*, 1984), and the SS-Tau, the same mixture without Tau, each compound at 10^{-3} M (total mixture molarity 1.4×10^{-2} M). For shipboard efficiency, these two solutions were prepared with artificial seawater before the cruise and frozen until used. The concentrations reported for the SS and SS-Tau mixtures are those of their components. All dilutions were made with SW.

Behavioral bioassay

The second pair of legs of the common gulf-weed shrimp have elongate, slender chelae (Fig. 1). On *Sargassum,* shrimp would occasionally bring the distal tip of one of the chelae to the mouth or bring the whole chela to the maxillipeds for grooming. We define these movements as chela flexion for the gulf-weed shrimp. Similar movements performed by the major claws of other decapod crustaceans can be induced by chemical stimuli (Case and Gwilliam, 1961; Field, 1977) and are defined as cheliped flexion (Field, 1977), but only one previous study has used this behavior to systematically assay chemical sensitivity (Trott and Robertson, 1984). In decapod crustaceans the chelate appendages are used generally in components of feeding and grooming behavior. For example, the lobster *Homarus americanus* uses the first two pairs of chelate legs for food recognition and feeding (Derby and Atema, 1982; Borroni *et al.*, 1985). The common gulf-weed shrimp uses the chelae to pick up foodstuffs such as bryozoans and hydroids when grazing on the *Sargassum* (Geiselman, in Butler *et al.*, 1983). Chela flexion was observed directly or under a dissecting microscope. Since it was occasionally difficult to distinguish chela movements to the mouth from chela movements to the maxillipeds, we include both as chela flexion. Consequently a few grooming movements may have contributed to our chela flexion rates. However, the mean spontaneous chela flexion rate (±S.D.) for the gulf-weed shrimp for all pre-stimulus periods was only 1.20 ± 0.95/min ($n = 195$). We initially determined that the frequency of chela flexion could be influenced by chemical stimuli; we then used it as a bioassay to examine the chemosensitivity of this shrimp.

Animals were placed in a crystallizing dish (76 mm in diameter and 37 mm deep) filled with 66 ml of seawater. After 15–30 min pre-test acclimation, the number of chela flexions was counted for 5 min before and for 5 min after the introduction of a test stimulus. The shrimp often showed a prolonged chela flexion in which the tip of the chela would repeatedly probe the mouth; however, to be scored as a separate flexion the chela had to be swung away from the side of the carapace at least 0.5 cm before its return to the mouth. A test stimulant (1 ml) was slowly injected from a hypodermic needle into the water near the shrimp's anterior end over a period of about 10 seconds. An injected stimulus would be maximally diluted to approximately 0.015 times if evenly mixed into the 66 ml of SW immersing the shrimp. The concentrations reported in the Results are injected concentrations, *i.e.,* not corrected for this dilution factor. The animal was exposed to actual concentrations between these two extremes. SW was injected to control for the effects of disturbances produced during stimulus delivery. Following each test, the dish was rinsed twice by gently pouring off and adding SW with the animal in place. The animals appeared remarkably undisturbed by this manipulation. At least 5 min passed before the next test began. An individual animal was tested once per day with either three different concentrations of the same compound or three different compounds and then returned to a holding bowl. The reported chela flexion score is the number of flexions observed post-stimulus minus the number observed pre-stimulus for each trial. All concentrations of the same test stimulus were presented in an ascending concentration series. Test compounds were considered to stimulate or inhibit chela flexion when post-stimulus flexion counts were significantly greater or smaller, respectively, than the counts for the pre-stimulus period (Wilcoxon Signed-Rank test for matched pairs, $P < 0.05$). The Mann-Whitney U Test compared chela flexion scores for significant differences between test stimuli ($P < 0.05$). The experiments were conducted with room temperature SW (20–22°C) between 0900 and 0200 hours under fluorescent lighting.

RESULTS

Description of behavior

The 35 shrimp used in these experiments ranged in carapace length from 2.7 to 7.6 mm (X ± S.D. = 5.5 ± 0.57); most were female (30) and half of these bore eggs. Prior to the injection of a test stimulus the shrimp would normally stand quietly, slightly tilted to one side with the legs spread and the large chelae extended forward. The animals made slow movements of the antennae and antennules. They frequently groomed the sides of the carapace, eyes, antennae, and antennules using the pair of small chelae on the first legs. The abdomen was often arched to bring the ventral surface of the tail underneath the head for grooming by the chelae on the first legs. This rest position was interrupted by locomotion around the perimeter of the dish. The animals often stopped perpendicular to the perimeter and probed the walls of the dish with the elongate chelae of the second legs. All behaviors were interrupted periodically by chela flexion. Activities after stimulus injection were similar except for increased locomotion and chela flexion in response to certain test stimuli. This led to our choice of chela flexion to quantify the behavioral responses.

Dependence of chela flexion rate on quantity and quality of chemical stimulus

Figure 2 shows the concentration-response relationship for the chela flexion scores in response to SS, Tau, Gluc, and Hyp. The chela flexion rates in response to the SS and Tau increased over 2 log concentration units. Injections of 10^{-3} *M* SS and 10^{-2} *M* SS and Tau significantly increased chela flexion rates. SS did not elicit a response at 10^{-4} *M* concentration; Tau was not tested at this concentration. The number of chela flexions after injections of Gluc and Hyp at 10^{-3} *M* and 10^{-2} *M* concentrations and SW were not significantly different from pre-stimulus values. Chela flexion in this shrimp is thus a chemically modulated behavior dependent on both the quantity and quality of the test stimulus.

Specificity of chela flexion

Figure 3 shows the specificity of chela flexion responses to the test stimuli in Table I. The only single compounds statistically effective in stimulating this behavior at 10^{-2} *M* were Tau, B-Ala, GSH, TMA, and Succ. Pro significantly reduced the spontaneous chela flexion rate. The other test compounds did not significantly affect chela flexions. Chela flexion scores for 10^{-2} *M* Tau and 10^{-2} *M* SS were not significantly different. Thus within the SS, Tau is the only significant single stimulant and it alone could account for the stimulatory ability of the SS mixture. At 10^{-3} *M* test concentration (see Fig. 3 inset) Tau, SS, and SS-Tau were all stimulatory, and their chela flexion scores were not significantly different from each other.

DISCUSSION

Our results identify some of the specific chemical cues for a component of feeding behavior in the common gulf-weed shrimp. The single compounds Tau, B-Ala, GSH,

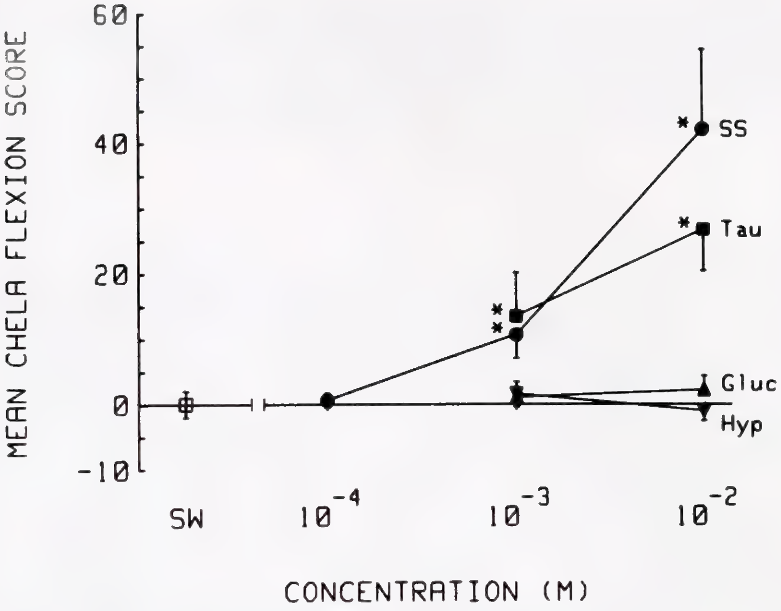

FIGURE 2. Concentration-response curves for chela flexion in *L. tenuicornis* in response to SS, Tau, Gluc, Hyp, and SW. The mean chela flexion score is the mean of the number of chela flexions/5 min after injection of a test stimulus minus the number of chela flexions/5 min before injection. Bars indicate the SEM when it is larger than the symbols. (*) The number of chela flexions during the post-stimulus period is significantly greater than the number of chela flexions during the pre-stimulus period ($P < 0.05$; Wilcoxon signed-ranks test for matched pairs, n = 10 for all SS trials; 5 for all other trials). The SS mixture concentration is designated by the molarity of its individual components. See Table I for key to abbreviations.

TMA, and Succ were all effective in eliciting chela flexion. The results also demonstrate that the SS mixture contained both stimulatory (Tau) and inhibitory (Pro) compounds. Tau alone could account for the SS mixture's stimulatory ability. However, the SS without Tau was also significantly stimulatory. The compounds stimulating chela flexion in this shrimp are generally those found to stimulate feeding behaviors in other crustaceans (for review see Ache, 1982; see also Trott and Robertson, 1984; Zimmer-Faust *et al.,* 1984; Carr and Derby, 1985). Chela flexion behavior was dependent not only on stimulus specificity but also on stimulus concentration, similar to results obtained for cheliped flexion in the ghost crab (Trott and Robertson, 1984) and for the functionally comparable "dactyl grasping" by the lobster (Borroni *et al.,* 1985).

The importance of Tau as a feeding stimulant for some littoral crustaceans and for the pelagic common gulf-weed shrimp may arise from its high signal to noise ratio in both littoral and pelagic environments, giving it excellent signal detection properties. Tau is a major free amino acid occurring in m*M* quantities in the tissues of some marine animals (Carter and Steele, 1982; Carr and Derby, 1985), and in tens of μm quantities in zooplankton (Jeffries, 1969); it is one of the main amino acids released by living zooplankton (Webb and Johannes, 1967). These or closely related animals may be natural food items for the common gulf-weed shrimp (Geiselman, in Butler *et al.,* 1983). In contrast to its high concentrations in prey tissues, Tau is present in low background concentrations of approximately 100 n*M* in inshore benthic waters (Davis *et al.,* 1985) and approximately 10 n*M* and lower in waters further offshore (Dawson and Gocke, 1978; Mopper and Lindroth, 1982). Tau concentrations within

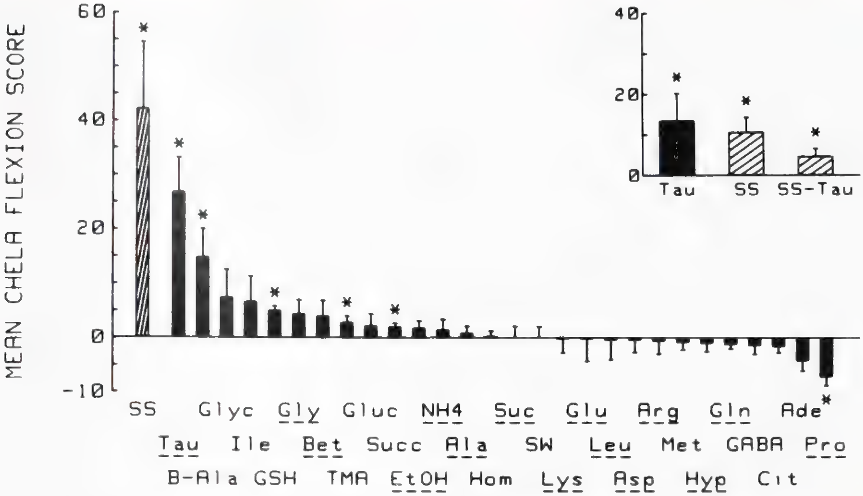

FIGURE 3. Chemical specificity of chela flexion in *L. tenuicornis*. The bars indicate the mean chela flexion score ± SEM (see Fig. 2 legend) for the test stimuli at 10^{-2} *M* concentration and, for Glyc, 4 g/l. Compounds with a dashed underline are components of the SS mixture. Inset: mean chela flexion scores ± SEM for Tau, SS and SS-Tau at 10^{-3} *M* concentration. (*) The number of chela flexions during the post-stimulus period is significantly larger or smaller than the number of chela flexions during the pre-stimulus period ($P < 0.05$; Wilcoxon signed-ranks test for matched pairs, $n = 10$ for all SS trials, 5 for all other trials including SS-Tau). Solid bars indicate single compound scores, hatched bars are scores for mixtures. Mixture concentrations are designated by the molarity of an individual component. See Table I for key to abbreviations.

the gulf-weed shrimp's microenvironment of *Sargassum* rafts are not known and may well be similar to inshore environments. These levels are at least one order of magnitude lower than those of most other free amino acids in both environments (Dawson and Gocke, 1978; Mopper and Lindroth, 1982; Davis *et al.,* 1985). Thus alone or within mixtures such as tissue fluids, Tau may have favorable signal to noise ratios of at least 10,000 or 100,000 at the source of release. Dilution away from the source would result in a ratio of about 10 at distances of centimeters to meters (Atema *et al.,* unpub.).

Crustaceans appear to have evolved specialized chemoreceptors to detect Tau. Electrophysiological studies have demonstrated receptors on the antennules of lobsters that are extremely sensitive to Tau, responding to concentrations as low as 10^{-13} *M* (Thompson and Ache, 1980). In addition, Tau receptors on the antennules and legs of lobsters have extremely restricted response spectra (Fuzessery *et al.,* 1978; Johnson and Atema, 1983; Johnson *et al.,* 1984). B-Ala, structurally very similar to Tau, is the second best stimulus for the Tau receptors of the spiny lobster (Fuzessery *et al.,* 1978) and in producing bradycardia in the kelp crab (Zimmer *et al.,* 1979). Since B-Ala was also our second best stimulus for chela flexion this behavioral response may be mediated by a Tau/B-Ala receptor population. This may imply that chemosensitivity to Tau/B-Ala in the common gulf-weed shrimp is mediated by chemoreceptors with similar characteristics as those described in littoral crustaceans.

Using cheliped flexion in the ghost crab as an assay for chemosensitivity, Trott and Robertson (1984) found that carbohydrates were effective stimuli. Carbohydrates were not effective stimuli for the common gulf-weed shrimp. These compounds have been found to stimulate feeding behaviors in only three other crustaceans: clearly in the semi-terrestrial fiddler crab (Robertson *et al.,* 1981), and less so in the marine porcelain crab (Hartmann and Hartmann, 1977) and antarctic krill (Hamner *et al.,* 1983). Carbohydrates would have a high signal to noise ratio for the semi-terrestrial ghost and fiddler crabs which feed on foods containing high levels of carbohydrate (Trott and Robertson, 1984) on sandy beaches which probably contain low carbohydrate backgrounds. Aquatic marine crustaceans on the other hand would find carbohydrates to be poor signals because they are generally present in high background concentrations in seawater (Burney and Sieburth, 1977); glucose for example is found in the micromolar range even in the Sargasso Sea (Liebezeit *et al.,* 1980).

Previous crustacean chemoreception studies have observed inhibition of filter feeding by single compounds (Hartmann and Hartmann, 1977) and reduction of other feeding behavior when certain compounds were added to a single stimulant or a stimulatory mixture (McLeese, 1970; Allison and Dorsett, 1977; Zimmer-Faust *et al.,* 1984; Borroni *et al.,* 1985). Our study shows that Pro has such inhibitory effects on the feeding behavior of the gulf-weed shrimp. Similarly in lobsters, Pro inhibited receptor cell responses to Hyp (Johnson and Atema, 1985) and antennule flicking responses (Spalding and Atema, 1983). Pro is found as a free amino acid in tissues from marine organisms in up to m*M* concentrations (Carr and Derby, 1985) and it is present in low background concentrations (1–100 n*M* range) in seawater (Daumas, 1976). Thus, like Tau, Pro may have good signal to noise properties. However, at present we do not understand its role in behavioral inhibition.

Since this behavior is common among decapod Crustacea, chela flexion can be used as a standardized assay to examine chemical feeding stimulants and inhibitors for higher crustaceans, concomitant with the measurement of the chemical noise levels in their environments. *Sargassum* crustaceans could continue profitably to be the focus of future studies comparing distantly related animals that share the same habitat and food items and closely related animals that inhabit different habitats. The shrimps *Latreutes* and *Hippolyte* and the crabs *Planes* and *Portunus* share the *Sargassum* habitat with the common gulf-weed shrimp and compete for the same food items such as polychaetes, flatworms, hydroids, bryozoans, and other crustaceans (Geiselman, in Butler *et al.,* 1983). The common gulf-weed shrimp also inhabits inshore vegetation and a very close relative, *Leander affinis,* is restricted to coastal areas (Holthuis, 1952). Such comparative studies of chemical feeding cues in crustaceans may provide insight into the phylogenetic and environmental constraints that determine the adequate stimuli for chemoreceptors. Perhaps evolutionary pathways could emerge that show the phylogenetic history of different chemoreceptors.

ACKNOWLEDGMENTS

We thank Dr. G. Harbison, chief scientist of *Oceanus* cruise #165 for shiptime, Dr. L. Madin for use of his lab space and equipment, C. Cetta and G. Dietzmann for shipboard help, and Dr. C. Lee for counsel on amino acid levels in seawater. We especially thank C. Diebel for inviting us to participate in the cruise and for encouragement and advice. T. Trott suggested and supplied some of the test chemicals. Dr. R. Voigt helped with the computer graphics and T. Trott and Drs. A. J. Tierney and R. Voigt criticized an earlier version of this manuscript. This work was supported by the Lerner-Gray Fund for Marine Research of the American Museum of Natural History, the Whitehall Foundation and NSF (BNS 8411969).

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SEX CHANGE AND LIFE HISTORY PATTERN IN THE SHRIMP *THOR MANNINGI* (DECAPODA: CARIDEA): A NOVEL CASE OF PARTIAL PROTANDRIC HERMAPHRODITISM

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ABSTRACT

A population of *Thor manningi* sampled for one year was composed of 50% primary males, 49% protandric hermaphrodites, and 1% primary females. Primary males have prehensile third pereiopods, massive appendices masculinae, and life-long sperm production. Protandric hermaphrodites pass first through a male phase (non-prehensile third pereiopods, small appendices masculinae, sperm production) and a transitional phase (reduction of appendices masculinae and sperm ducts, development of female incubatory flanges, ovarian development) before maturing into breeding (embryo-carrying) females.

Eighty-six to 100% of females (female-phase hermaphrodites and primary females) in monthly samples carried embryos. Breeding females produced new embryo clutches approximately every nine days. Although reproduction was continuous, recruitment chiefly occurred in the summer. Measurements of density, size-specific dry weight, and cohort analysis indicate average life spans of approximately 4–5 months, production of 35 mg dry weight/m^2/year, and a production:biomass ratio of 4.5.

Primary males do not occur in other protandric decapod species. It is hypothesized that *T. manningi* primary males persist at a high frequency in the population because they may be more efficient at fertilizing breeding females than are male-phase hermaphrodites.

INTRODUCTION

Protandric sex change has been reported in relatively few of the approximately 1800 species of caridean shrimps. Carpenter (1978), Subramonian (1981a), and Policansky (1982) have listed decapod crustacean species which have either been shown to be or are purported to be protandric hermaphrodites. Of the 32 decapod species reported as sex changers in these review articles, 26 are caridean shrimps. In the boreal caridean genera *Pandalus* and *Pandalopsis,* nearly all species show varying degrees of protandry (Berkeley, 1930; Butler, 1964, 1980; Hoffman, 1972; Charnov, 1979; Williams, 1984). In some species, all members of a population undergo a protandric sex change, *i.e.,* they reproduce first as males and then, with increasing age and size, become breeding females. In other species, not all individuals are sex changers: a variable proportion (<50%) of the population are primary females, individuals lacking male characters which breed only as females. Primary males, individuals breeding only as males throughout their lifetime, are unknown in protandric pandalid species. Fréchette *et al.* (1970) found a sexual system similar to that of pandalids in the crangonid *Argis dentata* Rathbun. In another well-documented case of protandry in the

Received 5 July 1985; accepted 25 November 1985.

Caridea, Dohrn (1950) found complete protandry in the hippolytid *Lysmata seticaudata* Risso, *i.e.*, all individuals function first as males before changing to females. In protandric carideans, the transformation from male to female is marked externally by loss of the appendices masculinae (shown to be copulatory structures by Bauer, 1976, and Berg and Sandifer, 1984) and by modifications of the endopods of the first pleopods (Dohrn, 1950; Butler, 1964, 1980; Charniaux-Cotton, 1975).

Chace (1972) raised the possibility of protandric hermaphroditism in the hippolytid genus *Thor*. In describing *Thor manningi*, Chace noted that the smaller size classes of a collection (made up of samples from many localities) were composed of males while larger size classes were dominated by mature females. His analysis of the situation was complicated by the occurrence of two types of males: "functional" males, individuals with prehensile third pereiopods and large appendices masculinae; and "nonfunctional" males, those with unmodified third pereiopods and small appendices masculinae. Chace hypothesized that the smaller "nonfunctional" males might represent males which had not yet reached maturity; large "nonfunctional" males might be males in the process of changing sex to female. With the limited museum collection available to him, Chace was unable to make a clear-cut decision on the problem of protandry which he recognized in *T. manningi*.

During a year-long sampling of macroinvertebrate epifauna in *Thalassia testudinum* Koenig seagrass meadows on the north coast of Puerto Rico (Bauer, 1985), several thousand *T. manningi* were collected. Observations and analyses of this material and laboratory studies on live shrimps have revealed the details of *T. manningi's* novel sexual system. The population sampled consists of 50% primary males which never change sex, nearly 50% protandric hermaphrodites which pass through a male phase (Chace's "nonfunctional" males) before maturing as breeding females, and a very small proportion of primary females, females which develop no male characters before reproducing as females. This report analyzes the sexual system of *T. manningi* and describes the changes in external and internal anatomy which support a conclusion of partial protandry in this species. The population structure, reproductive pattern, recruitment, life span, productivity, and other life history characteristics of this species are analyzed and reported.

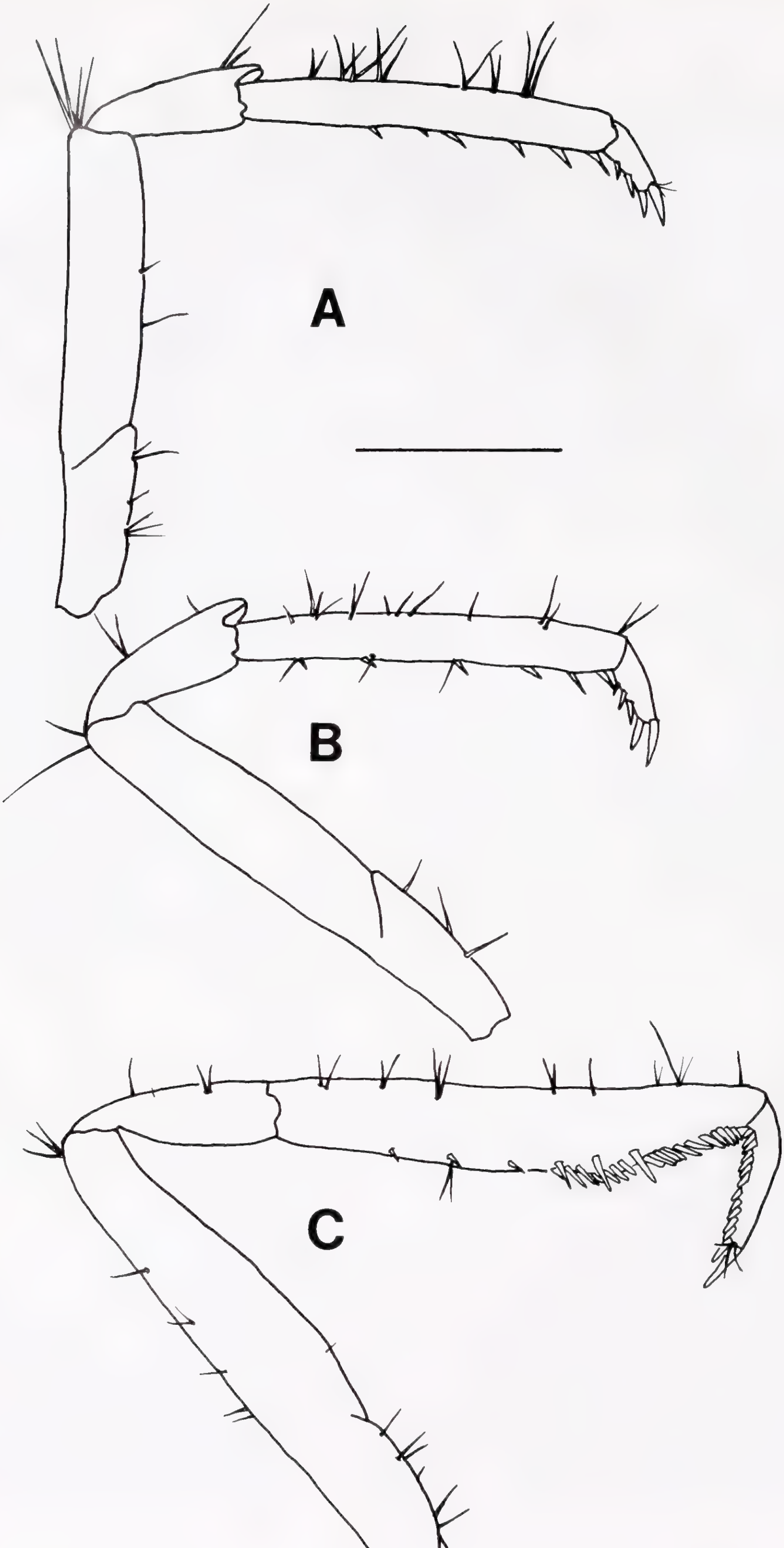
MATERIALS AND METHODS

The macroinvertebrate epifauna was sampled monthly from *Thalassia testudinum* meadows at Dorado, Puerto Rico (18° 29′N, 66° 15′W), from March, 1982 to February, 1983 (see Bauer, 1985, for details of habitat and sampling procedures). Samples were immediately preserved in 10% seawater formalin; invertebrates including *T. manningi* were sorted to species and placed in 70% ethanol for permanent storage. Densities of *T. manningi* reported here are based on random samples ($n = 20$ for 9 months, $n = 10$ for 3 months) taken at night with a 0.5 m wide pushnet using a 1.0 mm mesh liner. Some workers (Pihl and Rosenberg, 1982; Howard, 1984) have used enclosure trapping in sampling and estimation of shrimp densities in shallow water. In one set of night collections, I compared the efficiency of pushnet *versus* drop-trap (Pihl and Rosenberg, 1982) sampling in the estimation of absolute abundances. For *T. manningi*, there was no statistically significant difference in the mean number of individuals per square meter between pushnet samples (mean = 39.2, $n = 10$) and drop-trap samples (mean = 22.5, $n = 10$) (*t*-test, $P > 0.10$). In these seagrass meadows, pushnet sampling is as efficient as enclosure methods in estimating densities. Plankton tows taken at night through the seagrass with a 333 μm mesh net did not yield juveniles smaller than the smallest individuals collected with the 1.0 mm pushnet, *i.e.,* the entire size range of the *T. manningi* population was sampled by the pushnet.

Over 1100 females (hermaphrodites and primary females) and 1100 males (primary males) from monthly samples were examined, measured, and sexed in an analysis of monthly population structure. Before beginning routine measurements and sexing of material in monthly collections, extensive observations and dissections were done to understand sexuality in *T. manningi.* In this report, *mature* or *adult females* refers to females which were carrying embryos, or, rarely, those without embryos which showed no male characteristics and which were as large or larger than the smallest embryo-carrying (= ovigerous or brooding) female in that month's sample. Such mature females without embryos usually represent females captured after hatching of one brood but before spawning of the next. *Male-phase hermaphrodites* are individuals with non-prehensile third pereiopods which had appendices masculinae on the second pleopods. *Primary males* are individuals with prehensile third pereiopods. Shrimps in preserved samples sometimes had both third pereiopods missing, but primary males could be distinguished from male-phase hermaphrodites by the former's proportionately larger appendices masculinae, the long curved seta at the tip of the endopod of pleopod 1, and the absence of pleopod flanges (see description of appendage morphology in the Results section). However, only individuals with third pereiopods present were used in the morphometric measurements. Individuals lacking appendices masculinae which were smaller than the smallest ovigerous female but larger than the juvenile size range are termed *primary females.* Only 11 such individuals were observed in the samples. *Juveniles* are small individuals (0.5–1.0 mm carapace length) which could not be reliably sexed. Juveniles comprised from 0–17% (median = 6.5%) of the population in the monthly samples.

Carapace length (CL) is the distance from the posterior edge of the eye orbit to the posterior mid-dorsal edge of the carapace. In hermaphrodites and primary females, the developing ovary with its large yolky eggs can be observed easily through the transparent carapace with a stereomicroscope. Degree of ovarian development was characterized as: stage 1, no noticeable development; stage 2, vitellogenic oocytes distinct but ovary small; stage 3, ovary filling at least half the space above the cardiac stomach; stage 4, ovary completely filling that space. Embryos incubated by brooding females were classified with a system suggested by Allen (1966): stage 1, early embryos with no visible blastoderm; stage 2, blastoderm distinct, no eye development; stage 3, embryos with eyes, abdomen not free from cephalothorax; stage 4, embryos near to hatching, little or no yolk, large eyes, abdomen free from the cephalothorax. Determination of brood or clutch size was made by removing and counting embryos from incubating females. Only broods from females carrying stage 1 or 2 embryos were used; advanced embryos are more easily dislodged from females during sampling and subsequent handling.

Monthly size-frequency distributions with 0.1 mm size classes were constructed for females and primary males. Cohort analysis according to the methods of Harding (1949) and Cassie (1954) was used to identify and to separate overlapping cohorts, to obtain estimates of the mean size of individuals in a cohort, and to calculate the proportion of the total population comprised by a cohort. These are standard methods for analylzing field populations of caridean shrimps (Alon and Stancyk, 1982; Pihl and Rosenberg, 1982; Howard, 1984; Pihl and Pihl, 1984). For production and biomass calculations, the total population density was divided equally between males and females as the sex ratio was never statistically different from 1:1 (Chi-square analysis). The total male or female density was divided up among cohorts according to their


A
B
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proportions in the population. The relationship between dry weight and size (carapace length) was determined from freshly captured individuals which were measured, sexed, oven dried for 2 days at 60°C, and weighed on an analytical balance to the nearest 0.1 mg. The power curve $y = ax^b$ was used as the model for regressions of dry weight on size, where y is dry weight in mg, x is carapace length (mm), a is the y-intercept, and b is the slope. For primary males, the regression constants and explained variance (r^2) are $a = 0.89$, $b = 3.10$, and $r^2 = 0.69$ ($n = 30$); for females, $a = 0.69$, $b = 2.69$, and $r^2 = 0.83$ ($n = 41$). The biomass for males or females at a given sampling period was calculated by summing, for all cohorts, the products of weight of the average-sized individual in a cohort and the density of that cohort. The calculation of net productivity (P) of cohorts from one sampling period to the next was based on Crisp's (1971) growth increments method: $P = \sum_{1}^{i} \{(N1 + N2)/2\} (\Delta W)$, where N1 and N2 are density at the beginning and end of the period, W is the average weight of an individual in the cohort, and *i* is the number of cohorts present.

RESULTS

Secondary sexual characteristics of primary males and male-phase hermaphrodites

True or primary males are easily distinguished from male-phase female hermaphrodites by the structure of the third pereiopod (Fig. 1). In males, the distal end of the propodus is expanded and its lateral flexor margin is covered with long spatulate setae (Figs. 1C, 2A). In addition, the dactyl of pereiopod 3 in true males is elongate (compared to male-phase hermaphrodites and mature females) and similarly equipped with spatulate setae (Figs. 1C; 2A, B). The morphology of pereiopod 3 described for *Thor manningi* primary males occurs in males of some other caridean species (Chace, 1972; Butler, 1980) and has been termed "prehensile," and I will use the term in this report. By contrast, the third pereiopod of male-phase hermaphrodites is identical to that of mature females (Figs. 1A, B; 2C, D).

The endopod of pleopod 1 in primary males bears a series of spinulose to serrate setae on its medial edge, and the endopod terminates in a long curved seta (Figs. 3D; 4A, B). Endopods of male-phase hermaphrodites are variable in setation but generally bear medial setae which are more weakly spinulose than in true males. The long terminal endopod seta seen in primary males is much less developed in male-phase hermaphrodites (Fig. 3C). Pleopod 1 endopod structure in the few primary females collected is similar to that of male-phase hermaphrodites of similar size (Fig. 3B, C).

The medial anterior edge of pleopod 2 is the site of a copulatory structure, the appendix masculina (Figs. 4C; 5A, B, C, E). This process is naked proximally and setose distally. There are two basic setal types: (1) an inner group of strong, naked setae set in deep sockets and (2) a group of finely setulate setae which surround the inner stout naked setae (Fig. 4D). In individuals with prehensile third pereiopods, *i.e.*, primary males, the appendices masculinae are always massive in size, nearly as long as the endopods which bear them (Figs. 4C, 5E). In individuals with unmodified third pereiopods, the relative size of the appendices are smaller than in primary males (Figs. 4E; 5A-C). In primary males, the relative length of the appendix masculina appears to remain constant with an increase in male size (carapace length) while in male-phase

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FIGURE 1. Third pereiopods of *Thor manningi* sexual types, lateral view. A. Breeding female, 1.3 mm CL; B. Male-phase hermaphrodite, 1.3 mm CL; C. Primary male, 1.2 mm CL. Scale bar represents 0.5 mm.

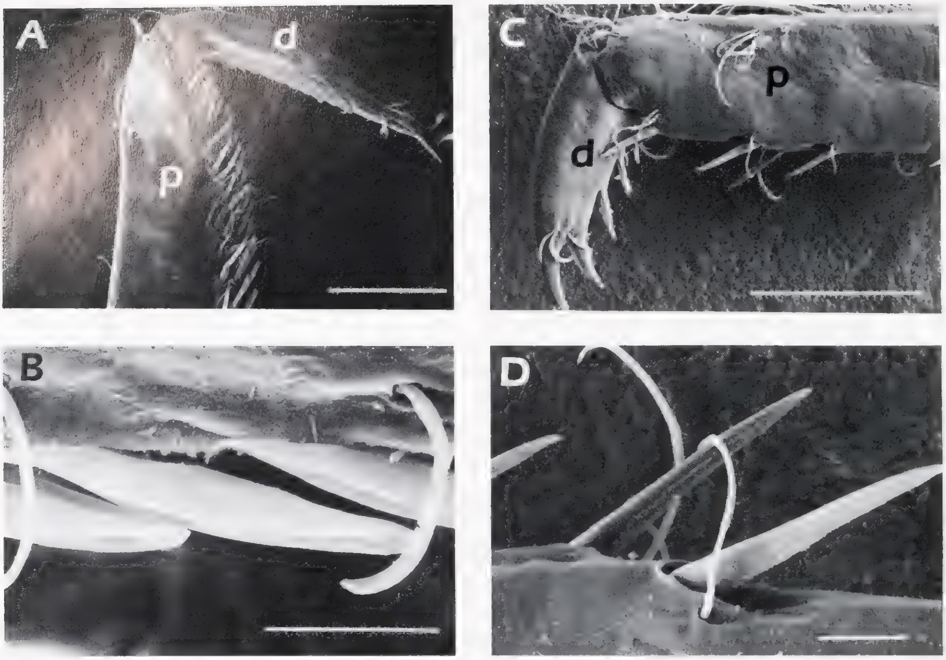


FIGURE 2. Structure of pereiopod 3 propodus and dactyl. A. Propodus and dactyl of primary male; B. Spatulate setae on the flexor margin of the dactyl shown in (A); C. Propodus and dactyl of a male-phase hermaphrodite; D. Setae from the flexor margin of the propodus in (C). p, propodus; d, dactyl; scale bar represents 200 μ.

hermaphrodites, the relative appendix masculina length decreases with increasing size (Fig. 6). This impression is confirmed by regression analysis of log (x + 1) transformed data (both variables in mm) with methods given in Kuris and Carlton (1977). The slope (b) of the regression line $\{P(H_0\text{: no regression}) < 0.0001\}$ for primary males is 0.90, indicating appendix masculina growth which is isometric or nearly so (Kuris and Carlton, 1977). For male-phase hermaphrodites, the slope of the regression line $\{P(H_0\text{: no regression}) < 0.0001\}$ is 0.20, confirming that appendix masculina growth is negatively allometric in this sexual type.

Another change in pleopod structure which accompanies growth in male-phase hermaphrodites is the development of a posterolateral flange, a thin non-muscular plate, on the basipod of (especially) the anterior three pairs of pleopods. This flange is always well-developed in mature females (Fig. 5D) and is associated with spawning and embryo incubation in many carideans (Hoglund, 1943; Bauer, 1976). In small male-phase hermaphrodites and in primary males of all sizes, the basipod is almost completely filled with muscle and there is no flange (Fig. 5A, E). A plot of flange (defined here as basipod lacking muscular tissue) width on carapace length (Fig. 7) shows that, after male-phase hermaphrodites (unmodified third pereiopod, appendices masculinae) reach 1.3–1.4 mm CL, the development of pleopod flanges is much greater than that of primary males (prehensile third pereiopods, appendices masculinae) in which the flange is virtually non-existent at any size. Again using log (x + 1) transformed variates, with both variables in mm, regression analysis confirms these conclusions. In male-phase hermaphrodites, the slope of the regression line is positive (b = 0.55)

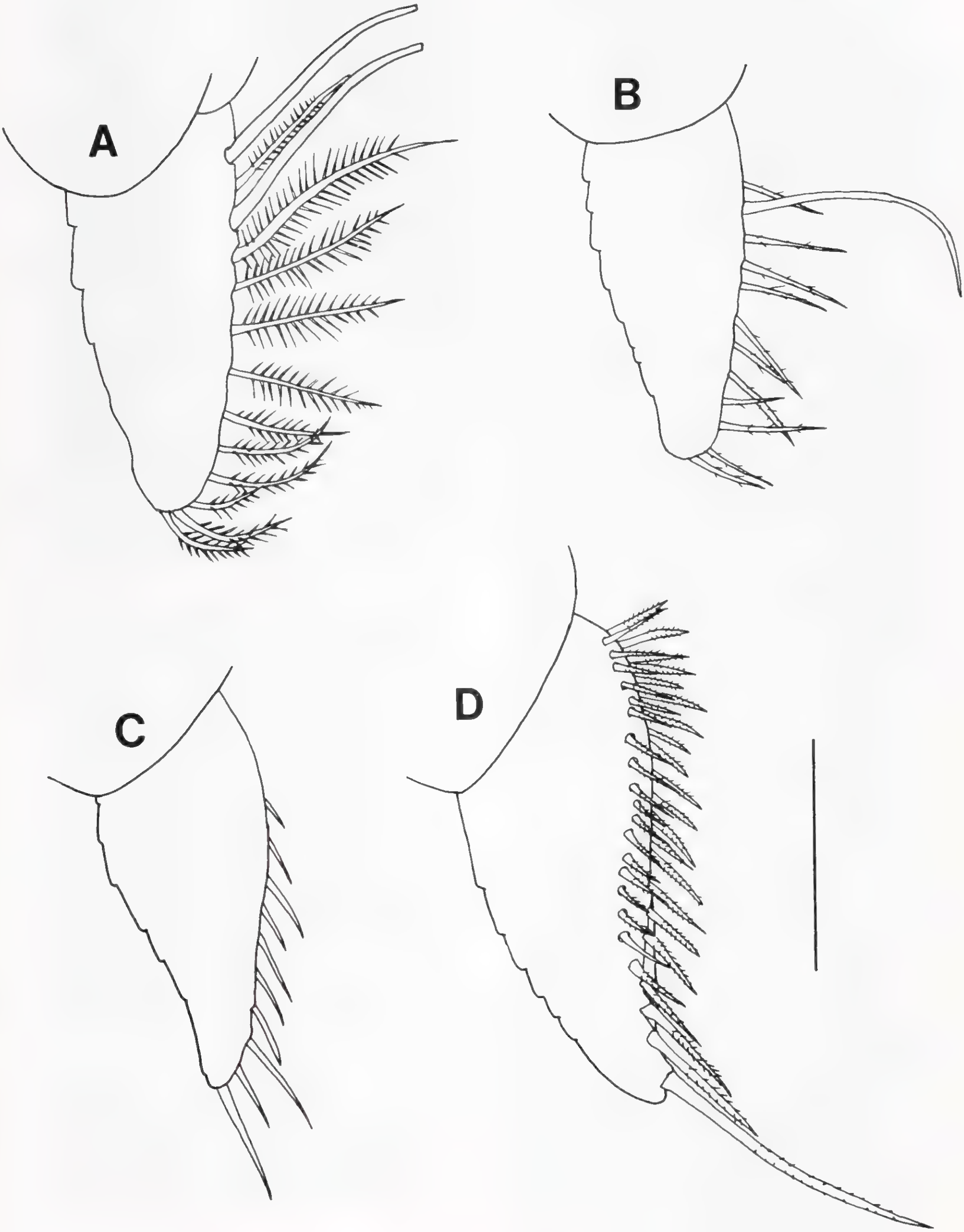
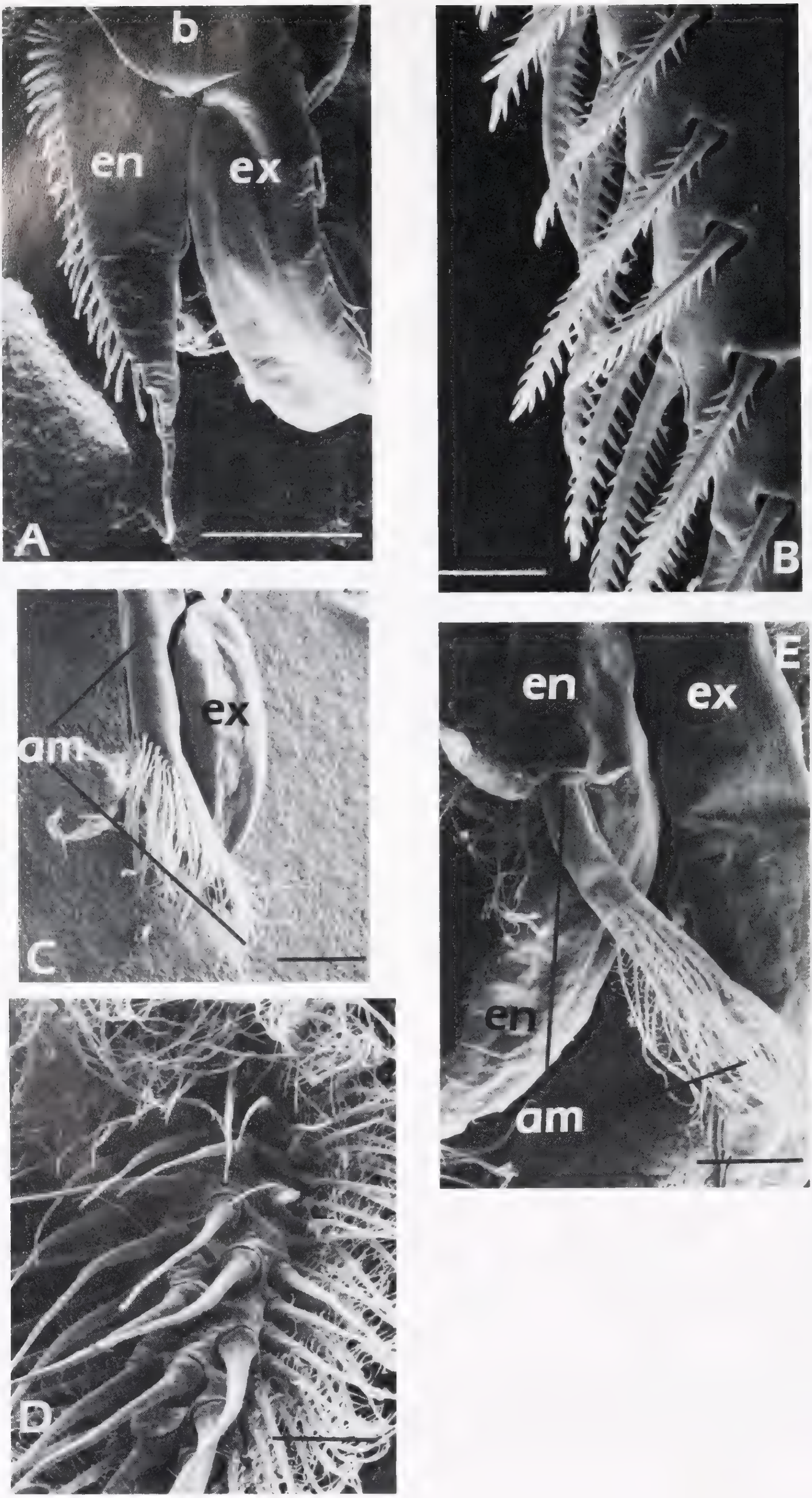


FIGURE 3. Endopods of the right first pleopods of different sexual types, anterior view. A. Breeding female, 1.3 mm CL; B. Primary female, 1.3 mm CL; C. male-phase hermaphrodite, 1.3 mm CL; D. Primary male, 1.2 mm CL. Scale bar represents 0.2 mm.

and the regression is statistically significant $\{P\ (H_0\text{: no regression}) < 0.0001\}$. For primary males, a calculated slope of a regression line is -0.003, *i.e.*, zero, and there is no significant regression $\{P\ (H_0\text{: no regression}) > 0.90\}$.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Changes in primary sexual characteristics in hermaphrodites

Thor manningi males and male-phase hermaphrodites carry sperm in the terminal end of the vas deferens, in structures termed the ejaculatory duct and bulb (Bauer, 1976; Fig. 8A). These latter structures are located in the cephalothorax just dorsal to the coxa of pereiopod 5. In *T. manningi,* a very short canal leads from the ejaculatory bulb to the male genital aperature on the proximal posterior face of the coxa. When the ejaculatory bulb and duct are developed and contain the bullet-shaped sperm of *T. manningi* (Fig. 8D), they can be observed through the body wall just above the pereiopod 5 coxa. From the smallest (0.8 mm CL) to the largest (1.7 mm CL) individuals recognizable as males by pereiopod 3 and pleopod 2 morphology, the ejaculatory bulb and duct are well developed and full of sperm (Fig. 8A, E, H). Dissections of the smallest recognizable male-phase hermaphrodites (0.7–0.9 mm CL) have never revealed sperm production, and sperm ducts, if observed, were rudiments lacking sperm. However, male-phase hermaphrodites larger than 1.0 mm CL always possessed an ejaculatory duct and bulb with some sperm. These structures and the vas deferens degenerate very rapidly with increasing size after 1.2 mm CL. The distal ends of the sperm ducts of three pairs of primary males and male-phase hermaphrodites of increasing size are compared in Figure 8. The sperm ducts of hermaphrodites 1.0–1.1 mm CL are similar in size and fullness of sperm as those of primary males of a similar size (Fig. 8A, B, C), indicating sperm production in the gonad. In hermaphrodites 1.3 mm CL, the vas deferens had degenerated to a narrow cord which lacks sperm (Fig. 8F), evidence of a cessation of sperm production (Fig. 8E, F). The ejaculatory bulb and duct still contain sperm. The gonad is a developing ovary filling with the large yolk-filled oocytes characteristic of carideans (Fig. 8G). There is an obvious relationship between the size of male-phase hermaphrodites and the degree of ovarian development (Table I). There is some vestige of the ejaculatory duct and bulb (with a very few sperm cells) in even the largest male-phase hermaphrodites (2.1 mm CL). In Figure 8I, these structures are shown for a hermaphrodite 1.6 mm CL; the gonad is a well developed ovary full of vitellogenic oocytes in these individuals (Fig. 8J). When hermaphrodites make the final transformation to the female-phase, *i.e.,* carry embryos, all male primary and secondary characteristics are lost. By contrast, with increasing size, the sperm ducts of primary males increase in size (Fig. 8A, E, H), are full of sperm, and the gonad shows testis structure typical of caridean shrimp.

Of over 1100 females (hermaphrodites and primary females) examined, only 11 could be clearly identified as primary females, *i.e.,* non-hermaphroditic females without any sign of primary or secondary male sexual characteristics. These were individuals in the same size range as male-phase hermaphrodites which lacked a prehensile pereiopod 3, showed development of pleopod flanges and/or ovarian development, and which showed no appendix masculina or sperm ducts. The number of primary females is probably underestimated since primary females cannot be distinguished from female-

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FIGURE 4. Sexual modifications of the anterior pleopods. A. Endopod and exopod of first pleopod of primary male, anterior view; scale bar, 200 μ; B. Serrate-spinulose setae on medial edge, endopod of pleopod 1, primary male; scale bar, 20 μ; C. Second pleopod of primary male, anterior view; black lines point out the base (upper line) and the setose tip of the massive appendix masculina (am) which completely covers the endopod anteriorly; scale bar in lower right corner, 200 μ; D. Medial view of setose tip of primary male appendix masculina, showing stout naked setae surrounded by finely plumose setae; scale bar, 50 μ; E. Anterior view of endopod and exopod, pleopod 2, of a male-phase hermaphrodite; black lines point out extent of the reduced appendix masculina (am). Note the small relative size of the appendix masculina of this specimen compared to that of the primary male in (C). Scale bar represents 100 μ. am, appendix masculina; b, basipod; en, endopod; ex, exopod.

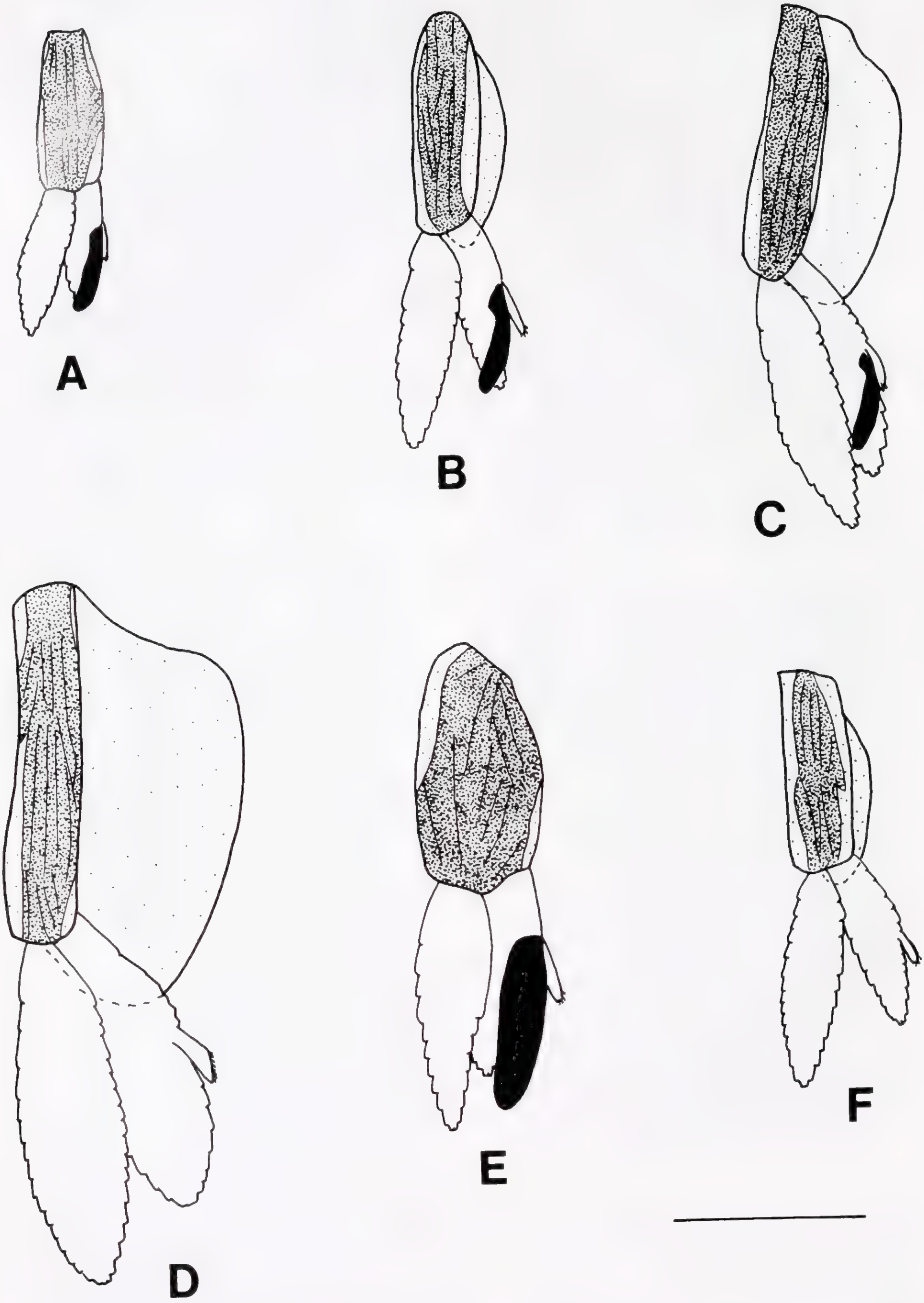

FIGURE 5. Diagrams of second pleopods of different sexual types, anterior view. A, B, C. From male-phase hermaphrodites of 1.0, 1.3, and 1.7 mm CL, respectively; D. Breeding female, 2.0 mm CL; E. Primary male, 1.3 mm CL, F. Primary female, 1.3 mm CL. The appendix masculina is shaded entirely black; heavy stippling represents basipod musculature; light stippling represents basipod free of musculature (basipod flange in B, C, D, and F). Scale bar represents 0.5 mm.

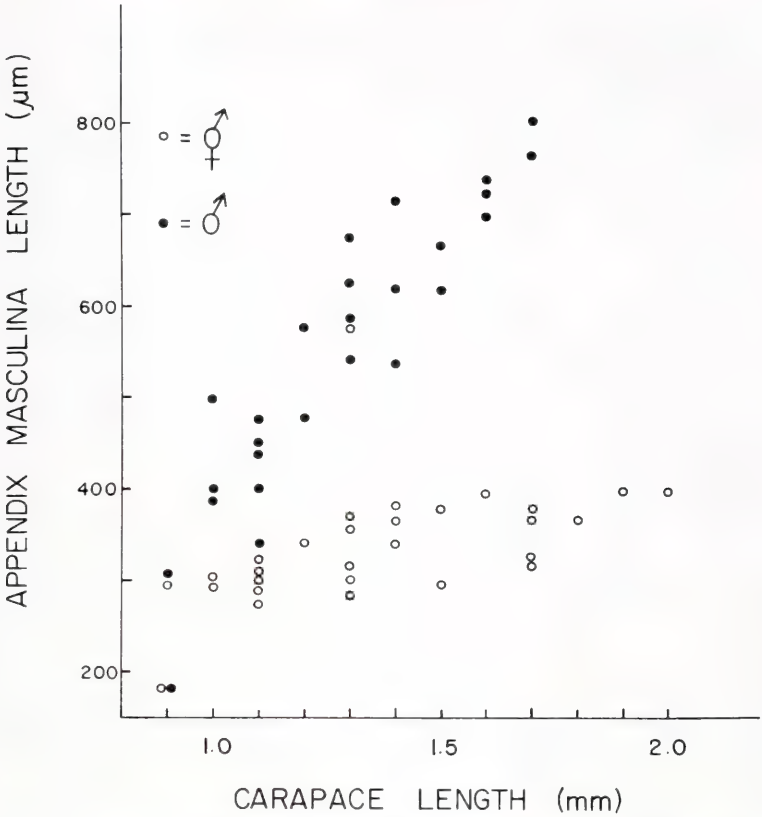

FIGURE 6. Variation of appendix masculina length with increasing size (carapace length) in primary males (dots) and male-phase hermaphrodites (circles).

phase hermaphrodites after the latter make the final change from male-phase hermaphrodite to ovigerous female. The low frequency of primary females in larger size groups may be inferred from their frequency in the smaller size groups (where they can be distinguished) assuming there is no differential mortality between male-phase hermaphrodites and immature primary females.

Size at sex change in hermaphrodites

The size of transformation from male-phase to embryo-carrying female-phase hermaphrodite was quite variable (Fig. 9). The size of the smallest ovigerous female varied in monthly samples 1.3–1.9 mm CL (median = 1.5 mm CL). On the other hand, the size of the largest male-phase hermaphrodite varied from 1.6–2.1 mm CL (median = 2.0). In some months of the study, there was considerable overlap between the male-phase hermaphrodite and mature female size-frequency distributions (Fig. 9). The fact that some male-phase hermaphrodites mature into mature females much more rapidly than most can be seen in Table I, where a small percentage of individuals in the smaller size classes do show distinct ovarian development. At the other extreme, a small percentage of the largest male-phase hermaphrodites show little or no ovarian

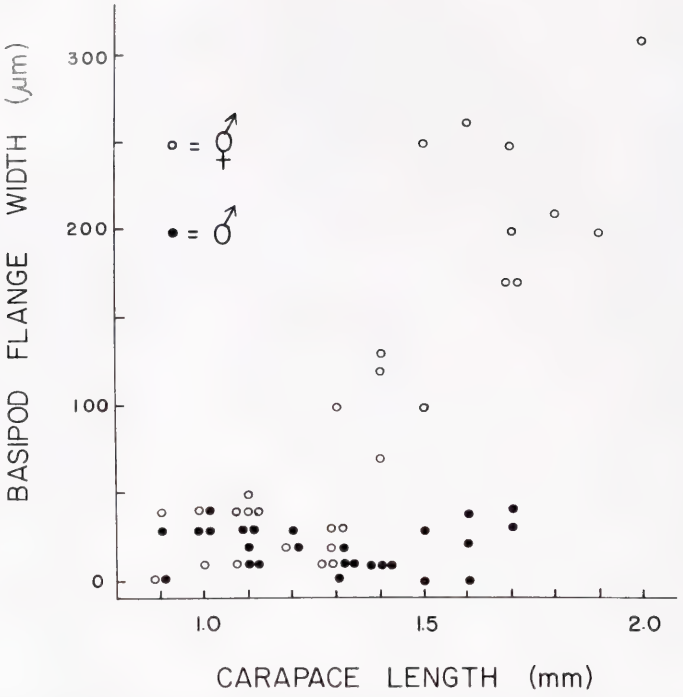

FIGURE 7. Variation of pleopod 2 basipod flange width with increasing size (carapace length) in primary males (black dots) and male phase hermaphrodites (open circles).

development, indicating a slow change in gonadal maturation to breeding female for some individuals.

Reproduction

Reproductive activity was high throughout the year (Fig. 9). The percentage of individuals with adult or mature female morphological characters which carried embryos varied between 86–100% in the monthly samples (median = 96%). Breeding females were iteroparous. At the same time incubated embryos were developing, the ovary filled again with vitellogenic oocytes. Figure 10 shows the positive correlation between stage of embryo development and stage of ovarian development. Females carrying recently spawned and fertilized embryos showed little ovarian development, while females with embryos near hatching had enlarged ovaries packed with yolk-filled egg cells. Laboratory observations indicate that within a day of embryo hatching, the female's post-hatch molt, mating, and spawning take place. Estimates of the duration of embryo incubation range from 6–10 days (median = 8 days, n = 20). These estimates are based on maintenance of captured females carrying recently spawned and fertilized embryos. The few females successfully mated in the laboratory showed incubation times within the range given above. Thus, the average adult female produces a new clutch of embryos approximately every nine days.

Clutch size varied from 22–147 embryos (median = 65) (Fig. 11). The regression line $Y = -95.3 + 83X$ describes the relationship between number of embryos per clutch (Y) and female carapace length (X). There is considerable variation ($r^2 = .33$; $n = 54$) in clutch size with increasing female size, *i.e.*, some large females had lower than expected broods (Fig. 11). In studies on the caridean *Heptacarpus pictus* Stimpson (Bauer, 1976), I observed that females breeding for the first time had small broods for their size (unpub. obs.). The large *T. manningi* females with low clutch sizes might be those females which completed sex change at a large size and thus were producing their first brood.

Recruitment and life span

Episodes of recruitment, *i.e.*, the appearance of a cohort of individuals in the smaller size classes, occurred several times throughout the year (Fig. 9). However, the strength of recruitment fluctuated greatly among these episodes. The monthly abundance of *T. manningi* varied seasonally, with highs from May–September (Fig. 12). These higher abundances were due to heavy recruitment in May, June, and August (male and female cohorts b, c; Fig. 9). A high proportion of individuals in samples from these months were in the smallest size classes; individuals too immature to sex accurately comprised large percentages (15–18%) of the samples. Thus, the high densities of *T. manningi* from July–September resulted from the strong pulses of recruitment in May, June, and August.

The life span of the "average" individual in a cohort on the seagrass meadows was estimated as the number of months the cohort was recognizable in the size-frequency distributions. Since separation of cohorts by the probability paper methods used is not precise, the following estimates of longevity should be viewed as approximations. Cohorts b and c (Fig. 9) were clearly recruited and subsequently disappeared during the sampling period. In females (hermaphrodites and primary females), both these cohorts survived 5 months; in males, cohorts b and c had apparent longevities of 4 and 3 months, respectively. Cohort a, occupying the smallest size classes in March, probably was recruited that month and thus females survived at least 5 months and males at least three months. Cohort d was recruited in October and had nearly died out by February, *i.e.*, a probable life span of 5 months. In summary, the median cohort longevity was 5 months for females and 3.5 months for males. The discrepancy between males and females might reflect a true difference in length of life. However, the size range of primary males is less than that of females, and a lower resolution of overlapping cohorts in males might be responsible for a lower estimate of their life span.

Maturation

When female cohorts were first sampled, the average-sized individual was near or at the size (1.0 mm CL) at which most individuals are male-phase hermaphrodites (Fig. 9). Approximately two months later, the "average" individual in the cohort reached mature female size (larger than the smallest ovigerous female in a particular month). In female cohorts a and d, the average-sized female was then breeding as a female for three months; in cohorts b and c, this period was about 2 months. Thus, breeding females were capable of producing 6–10 clutches of embryos during their lifetime, given the constant iteroparous reproduction discussed earlier and 9 days between spawnings. The mean size of individuals in male cohorts was always equal to or greater than the minimum size (0.8–0.9 mm CL) at which primary males have copulatory appendices and sperm in the sperm ducts. Primary males were thus reproductively mature by the time they were first sampled after recruitment, *i.e.*, within a month or less after settling from the plankton.

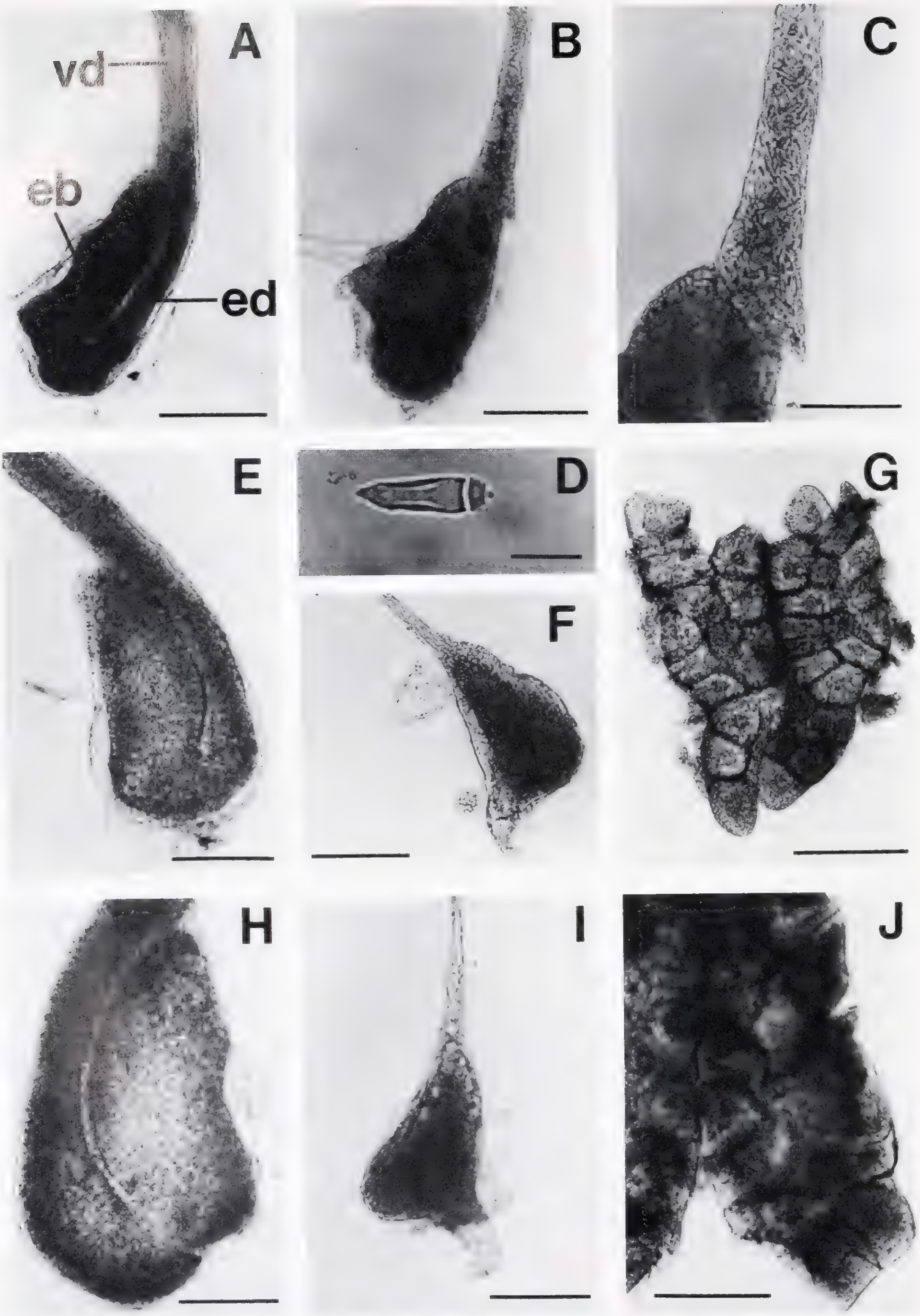

FIGURE 8. Primary sexual characteristics of *Thor manningi* sexual types. A. Terminal end of sperm duct, primary male, 1.1 mm CL; scale bar, 200 μ. B. End of sperm duct in male-phase hermaphrodite, 1.1 mm CL; scale bar, 200 μ. C. Vas deferens full of rod-like sperm cells; scale bar, 100 μ. D. Sperm cell; scale

TABLE I

Degree of ovarian development in Thor manningi *male-phase hermaphrodites**

Size Class	% Individuals with ovary in developmental stage: Stage 1	Stage 2	Stage 3	Stage 4	n
0.70–0.79	100	0	0	0	2
0.80–0.89	94	6	0	0	33
0.90–0.99	96	2	2	0	72
1.00–1.09	97	3	0	0	59
1.10–1.19	98	0	2	0	48
1.20–1.29	98	2	0	0	54
1.30–1.39	80	11	7	2	45
1.40–1.49	86	5	9	0	21
1.50–1.59	50	21	29	0	34
1.60–1.69	27	30	30	13	30
1.70–1.79	25	50	21	4	24
1.80–1.89	20	10	0	70	10
1.90–1.99	18	18	45	18	11
2.00–2.10	0	50	25	25	4

* Stage 1, no noticeable development; Stage 2, vitellogenic oocytes distinct but ovary small; Stage 3, ovary filling at least one-half the space above the caridac stomach; Stage 4, ovary completely filling space above the caridac stomach. Male-phase hermaphrodites are grouped in 0.1 mm CL size classes. n, number of individuals examined in each size class.

Productivity

The net secondary productivity, estimated by the growth increments method (Crisp, 1971), was 34.7 mg dry weight/m^2 for the 11.6 month period for which it could be calculated. Females (hermaphrodites and primary females) accounted for 63% of this production as opposed to 37% by the smaller males. The average annual biomass of the *T. manningi* population at the Dorado meadows was 8.4 mg dry weight/m^2. Using a value for production extrapolated to 12 months from the 11.6 month period covered in sampling, the production to average annual biomass ratio for this population is estimated to be 4.5. This population was thus replacing its average biomass every 2.7 months (turnover time).

DISCUSSION

Ghiselin (1969) proposed a size advantage model as one explanation for the evolution of protandric hermaphroditism. This model was further amplified by Warner (1979), Charnov (1982), and discussed in various reviews such as Policansky (1982). According to this hypothesis, protandry is favored in a species in which reproductive fitness is positively correlated with size in females but not in males. There is a positive correlation between clutch size and body size in *T. manningi* females (however, this is generally true of gonochoristic caridean shrimp species as well). *Thor manningi*

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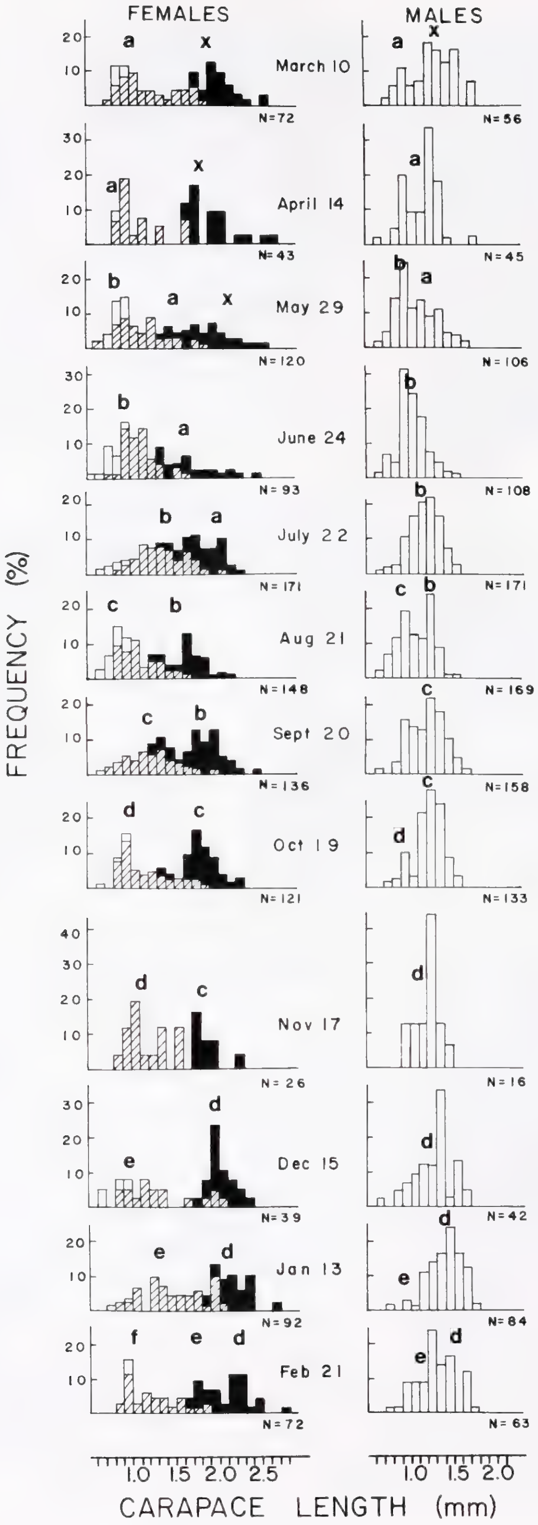
bar, 10 μ. E. End of sperm duct, primary male, 1.3 mm CL; scale bar, 200 μ. F. End of sperm duct of male-phase hermaphrodite, 1.3 mm CL; scale bar, 200 μ. G. Ovary of the male-phase hermaphrodite whose reduced sperm duct is shown in (F); note vitellogenic oocytes; scale bar, 500 μ. H. End of sperm duct of a primary male, 1.5 mm CL; scale bar, 200 μ. I. End of sperm duct of male-phase hermaphrodite 1.6 mm CL; scale bar, 200 μ. J. Ovary of male-phase hermaphrodite whose reduced sperm duct is shown in (I); eb, ejaculatory bulb; ed, ejaculatory duct; vd, was deferens, scale bar, 500 μ.


FEMALES
MALES
FREQUENCY (%)
March 10
N=72
N=56
April 14
N=43
N=45
May 29
N=120
N=106
June 24
N=93
N=108
July 22
N=171
N=171
Aug 21
N=148
N=169
Sept 20
N=136
N=158
Oct 19
N=121
N=133
Nov 17
N=26
N=16
Dec 15
N=39
N=42
Jan 13
N=92
N=84
Feb 21
N=72
N=63
a
x
b
c
d
e
f
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20
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40
1.0
1.5
2.0
2.5
CARAPACE LENGTH (mm)

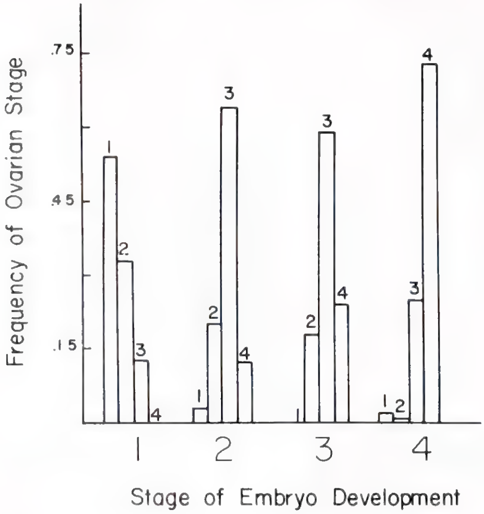

FIGURE 10. Degree of ovarian development in females carrying embryos at early (no noticeable blastodisc, stage 1) to late (nearly hatching, stage 4) stages of development. Stages of ovarian development are written above their histogram bars and range from stage 1 (no noticeable ovarian development) to stage 4 (maximum ovarian fullness). Numbers of females examined with embryos at stage 1, 213; stage 2, 64; stage 3, 34; stage 4, 95.

primary males are smaller than mature females and the evidence available indicates that male reproductive success is not correlated with size. Sufficient sperm production for insemination of the largest female clutches begins at a very small size in primary males. Observations on *T. manningi* in laboratory aquaria give no indication of male-female pair formation, male defense of a territory, fighting between males or other conditions which would favor large male size. This species is very similar in overall morphology and behavior to other hippolytids such as *Heptacarpus pictus* and *H. paludicola,* and mating in these species (Bauer, 1976; 1979), as in many carideans with disparate male-female size, is a brief encounter without complex precopulatory behavior (in which a larger male size could be an advantage).

Given the above, protandry in *T. manningi* and similar species should be advantageous. Females have to pass through a size range at which they are not large enough to produce embryos. It is the same size range at which primary males can produce sperm and fertilize females. It appears that in *T. manningi* females there has been selection for development of male characteristics during their growth (juvenile stage) to minimum breeding female size. Such females, now protandric hermaphrodites as a result of this selection, have increased their total reproductive output, even if they mate just once (as a male) with an adult female (female-phase hermaphrodite or primary

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FIGURE 9. Monthly size-frequency distributions of *Thor manningi* females (= male-phase hermaphrodites, female-phase hermaphrodites, and primary females) and males (= primary males). In the female distributions, histogram bars with diagonal lines represent male-phase hermaphrodites; shaded bars mature females; primary females which could be identified as such (n = 11) were too few to represent separately from male-phase hermaphrodites in the figure; unshaded bars, juveniles. Juveniles are not separated from mature males in the male histograms. Juveniles were individuals which could not be reliably sexed and were distributed between males and females at random (see Materials and Methods). Lower case letters represent cohorts, and are placed to show the mean size of individuals in that cohort for a particular month.

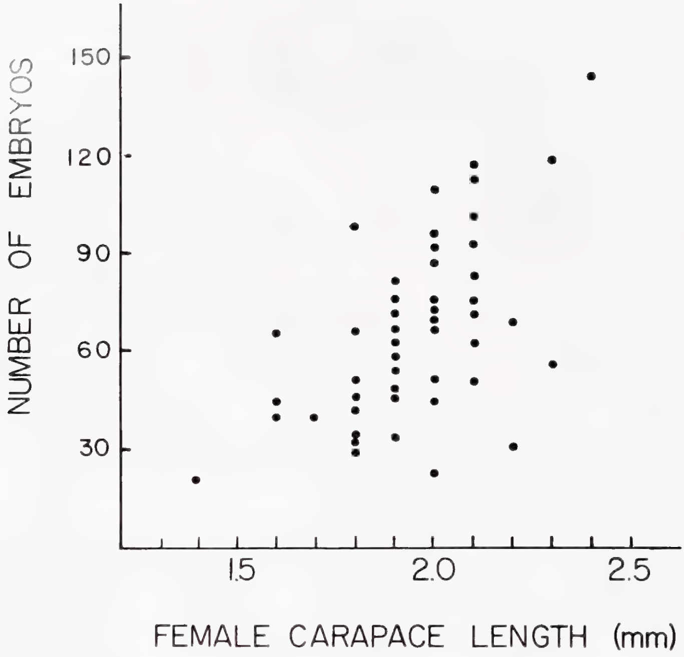

FIGURE 11. Variation of clutch size (number of embryos carried) with female size (carapace length).

female). It is easy to propose this scenario but the disturbing question (*cf.*, Policansky, 1982) is: why have not more caridean species with similar small male:large female size-frequency distributions evolved protandry as in *T. manningi?* There are many hippolytid species with small males and large females. The size-frequency distributions of males and females in the hippolytid *Heptacarpus pictus* showed no overlap at the beginning of the winter breeding season (Bauer, 1976) but sampling throughout the year and examination of sexual characters demonstrated that this was due to differential growth, not protandry. The only well-studied case of protandry described in the Hippolytidae is that of *Lysmata seticaudata* (Dohrn, 1950; Charniaux-Cotton, 1975).

Although protandry is not common in the Hippolytidae, it is usual in the pandalid genera *Pandalus* and *Pandalopsis* (Butler, 1980; Williams, 1984). In these genera, some species are completely protandric *i.e.*, all individuals in the population function first as males, then as females. In other pandalid species, the majority of individuals are protandric while the rest of the population are primary females which do not pass through a male phase. The proportion of primary females in these pandalids varies from a few percent to nearly (but always less than) 50% of the population (Charnov, 1979, 1982). There are *never* primary males in pandalid species with protandry. However, there are some pandalids, *e.g.*, *Pandalus bonnieri* (Hoffman, 1972) and *P. propinquus* (Williams, 1984) which are completely dioecious with primary males and primary females. Fréchette *et al* (1970) reported a protandric system with primary females in the crangonid *Argis dentata,* while Noel (1973) described an apparently similar situation in the processid *Procesa edulis.* In the hippolytid *Lysmata seticaudata,* all individuals are protandric (Dohrn, 1950). Subramoniam (1981b) described the sexual system of the sand crab *Emerita asiatica* as one in which the population is 50%

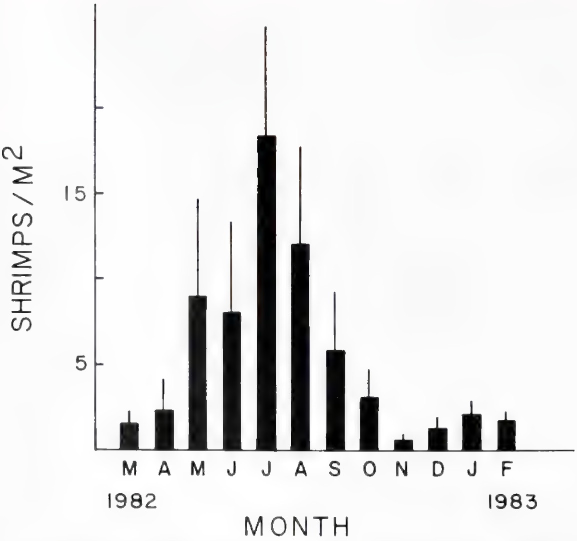

FIGURE 12. Monthly abundances (densities) of *Thor manningi* at Dorado, Puerto Rico, seagrass meadows. Bars are mean number of individuals per square meter, vertical lines above the bars are the upper 95% confidence intervals.

protandric, 50% primary female. *Thor manningi* appears to be unique among decapod crustaceans exhibiting protandric hermaphroditism in that there are primary males; in fact, 50% of the population studied were primary males.

An intriguing question arises: why are there primary males in *T. manningi* populations? Permanent males do not exist in other protandric decapod species. Based on the size advantage model and Charnov's (1982) hypotheses on sex allocation, one might predict that sexual systems with only protandric hermaphrodites or a mix of hermaphrodites and primary females would supplant those with primary males. However, in the *T. manningi* sexual system, primary males may be maintaining themselves genetically in the population by being more efficient inseminators of breeding females than the male-phase hermaphrodites. There is evidence that this is true in *T. manningi*. Primary males produce sperm very soon after arriving on the seagrass meadows and continue to do so throughout their lives. In contrast, male-phase hermaphrodites actively produce sperm only for a brief period, when they are between 1.0–1.2 mm CL. Although larger male-phase hermaphrodites no longer produce sperm, they invariably have a small packet of sperm in the persisting ejaculatory duct. This may indicate that they do not mate as males after gonadal sperm production stops. If they did, one would expect to find some of these larger male-phase hermaphrodites with empty ejaculatory ducts, *i.e.*, sperm has been discharged in mating. In addition, these large male-phase hermaphrodites have copulatory structures (appendices masculinae) which are reduced in size. Primary males, on the other hand, have enlarged appendices masculinae and the third pereiopod is prehensile, perhaps an adaptation for mating (seizing the female). In summary, I hypothesize that primary males fertilize a higher percentage of embryo clutches (produced by the iteoparous females) than do male-phase hermaphrodites and, for that reason, primary males continue to persist so successfully in *T. manningi* populations. Experiments on the mating success of primary males and male-phase hermaphrodites (of various sizes) must be done in order to test this hypothesis.

ACKNOWLEDGMENTS

My heartfelt thanks to Grace Palmer for her meticulous work in recording and compiling observations for the size frequency distributions. I gratefully acknowledge Dr. Zvi Weisz for the use of scanning electron microscope facilities, and my thanks to Oscar Resto and Carlos Rivera for thier help with preparation and examination of samples. Funds for student help and supplies for this investigation were provided by a FIPI grant from the Department of Academic Affairs, University of Puerto Rico, Rio Piedras. My thanks to Drs. Frank Ferrari, Bradford Hawkins, Robert Warner, and the anonymous reviewers for thier editorial assistance with the manuscript. This report is Contribution No. 1 of the Center for Crustacean Research, University of Southwestern Louisiana.

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SEXUAL REPRODUCTION OF A SOFT CORAL: SYNCHRONOUS AND BRIEF ANNUAL SPAWNING OF *SARCOPHYTON GLAUCUM* (QUOY & GAIMARD, 1833)

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ABSTRACT

The sexual reproduction of the alcyonacean octocoral *Sarcophyton glaucum* (Quoy & Gaimard, 1833) was studied for a period of about four years on the coral reefs of the northern Red Sea. *S. glaucum* is a dioecious species with gonads borne in the autozooids. The smallest colonies bearing testes measure 11 cm^3 (6–7 years old) while females attain maturity at a much larger colony size of at least 61 cm^3 (> ten years old). Sex ratio of the population is 1:1. The annual development of the sperm sacs takes 10–12 months. Oogenesis occurs every year, however egg maturation requires 22–23 months, resulting in the presence of two cohorts of oocytes in each female. The mature eggs are large with a maximal diameter of 500–750 μm. *S. glaucum* has a brief annual spawning period which occurs in the majority of the population during a single night (in 1980, spawning was on 9 July). The large size of the eggs is not the ultimate cause for their prolonged period of oogenesis. Nevertheless, the synchronous maturation of numerous eggs produced by a colony during a brief spawning period demands high energy expenditure which is allocated during two years. Fertilization is external and fully developed planulae are obtained 36 h after spawning. The larvae swim actively for 14 days, hence, promoting wide dispersal of the species. Life history features of *S. glaucum* include large body size, late age at the onset of reproduction, prolonged oogenic cycle, slow growth of the colony, and long life span.

INTRODUCTION

Anthozoans are capable of reproducing sexually in a variety of ways (Campbell, 1974; Chia, 1976; Fadlallah, 1983). Little attention has been paid to the sexual biology of soft corals (Octocorallia: Alcyonacea). Some studies have focused on planulating species of the family Xeniidae (Gohar, 1940; Benayahu and Loya, 1984a, b). Among members of the family Alcyoniidae several modes of sexual reproduction have been recorded. The temperate species *Alcyonium digitatum releases gametes, and A. hibernicum* has internal parthenogenesis of planular development (Hartnoll, 1977). Although alcyoniids are very common on coral reefs, little is known about their sexual reproduction and gonadal development. Yamazato *et al.* (1981) present circumstantial evidence for gamete spawning in *Lobophytum crassum,* and an external mode of planular brooding was described in the alcyoniid *Parerythropodium fulvum fulvum* (Benayahu and Loya, 1983). Moreover, recently spawned eggs were collected from several alcyoniids in the Great Barrier Reef, Australia (Bowden *et al.* 1985).

Among the Red Sea soft corals, the alcyoniids are very abundant and constitute 52% of the total number of soft coral species (Benayahu, in prep.). *Sarcophyton glaucum* (Fig. 1) is one of the most conspicuous alcyoniids on the Red Sea reefs and at numerous

Received 28 August 1985; accepted 25 November 1985.

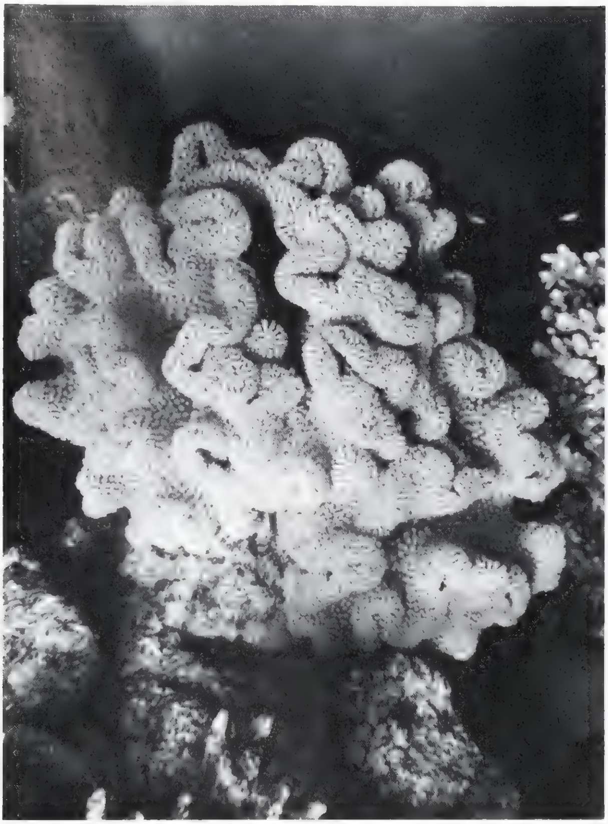

FIGURE 1. A colony of *Sarcophyton glaucum*.

sites across the Indo-West Pacific Ocean (Verseveldt, 1982). Early studies on soft corals deal with the morphology and anatomy of *S. glaucum* (Moseley and Oxon, 1876; Pratt, 1903); however, virtually nothing is known about its life history. This paper examines various aspects of sexual reproduction in *S. glaucum*. We present the annual cycle of gonadal development, population sex-ratio, size at the onset of reproduction, mode of reproduction, and its annual duration. In addition, we report for the first time direct evidence for a synchronous and brief annual gamete release of a soft coral.

MATERIALS AND METHODS

The study was conducted at two reef locations in the northern Gulf of Eilat. One site was Muqebla', 12 km south of Eilat, where the shallow *Sarcophyton* population (3–5 m) was studied. The deep water population (27–30 m) was examined near the Marine Laboratory of Eilat (M.B.L.). The two populations were studied from October 1977 to July 1981. Almost every month fragments of 10–12 cm^2 each were cut out from the disk of 10–20 large colonies, sampled in both locations. Additionally, 10 large colonies were numbered by plastic tags in each depth, and were sampled monthly to follow gonadal development within the same colony. All samples were preserved in 4% formalin in seawater.

The polyp cavities of the samples were examined with a binocular stereoscope for genital development and sex determination. Wet mounts of septa from each fragment were examined microscopically; the diameter of oocytes and sperm sacs was measured with a micrometer. Oocytes were scanned by electron microscopy following the method of Benayahu and Loya (1983).

To determine the relationship between colony size and the onset of sexual maturity, colonies of various sizes were collected prior to each breeding season (1978–1980). Formalin fixation of these colonies caused their complete contraction. Water displacement from a graduated cylinder was used to determine the volume of each colony (for further details see Benayahu and Loya, 1984a). These colonies were sectioned with a scalpel, and their sexual condition was recorded.

Spawning of *S. glaucum* was followed during June–August 1980. Colonies were maintained in 0.4 m^3 tanks filled with filtered running seawater. Plankton nets filtered the run-off from the outlets. After spawning the fertilized eggs were collected and reared into planulae in aerated seawater aquaria (25°C). In addition, during the summer of 1980, sequential underwater examination of gonads was performed in hundreds of colonies. Spawning in the field along a depth gradient (0–35 m) was inferred by the disapperance of mature oocytes or sperm sacs.

RESULTS

Gonads

S. glaucum is a dioecious soft coral. The gonads of both sexes develop in the autozooids on the four lateral and the two ventral mesenteries. Gonads are borne along the entire length of the polyp cavities. The number of oocytes or sperm sacs produced by each polyp is determined by its cavity length. In large colonies such a cavity can exceed 10–15 cm. One cm of a cavity contains as many as 25–35 oocytes or 50–70 sperm sacs. Prior to the breeding season (see below) the gonads fill most of the available space of the autozooids (Fig. 2a, b). Young and sexually immature polyps are located along the margins of the colony.

The spherical oocytes are an opaque cream color, and a few weeks before ripening they become pink. The sperm sacs are rounded, transparent, and white. Therefore, even in the field, sex is easily determined by these colors. After preservation in alcohol or formalin the gonads become yellow. Size measurements of the oocytes and the sperm sacs indicate a marked synchronization in the reproductive state of different polyps within a colony. No sex changes were detected during the study within the 20 tagged colonies.

Size at sexual maturity and sex ratio

Examination of 186 colonies of various size groups indicates that all specimens smaller than 10 cm^3 are sexually immature. The smallest testes-producing males are at least 11 cm^3. Females attain maturity at a much larger colony size of 61 cm^3. Most of the large colonies (>61 cm^3) are fertile and only a small percentage of the population lacks gonads.

Sex ratio within the population was obtained from the regular monthly samples throughout the study. In the shallow reef (3–5 m) 267 colonies were examined, of which 141 were females and 126 males. A total of 243 colonies sampled at the deep reef (27–30 m) resulted in 136 females and 107 males. No significant deviation from a 1:1 sex ratio in both reef zones was found (χ^2 test, $P > 0.05$).

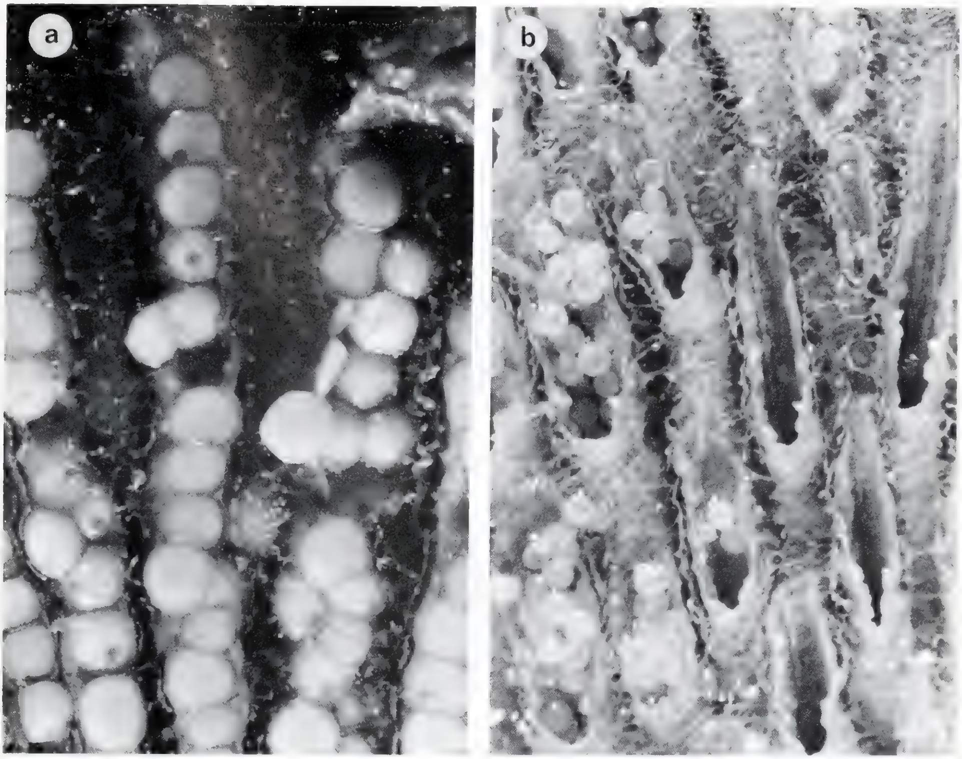

FIGURE 2. *Sarcophyton glaucum* a. Oocytes in polyp cavities of a sectioned colony (×25). b. Sperm sacs in polyp cavities of a sectioned colony (×100).

Annual cycle of gonadal development

Figure 3 demonstrates the monthly changes in the mean maximal diameters of sperm sacs in shallow water (Fig. 3a) and in deep water (Fig. 3b). Young testes (80 μm) are found in 3–5 m depth during August–September (Fig. 3a) and in 27–30 m during September–October (Fig. 3b). Their build-up generally takes 10–12 months. The testes' common diameter is approximately 400 μm prior to spawning.

Examination of female colonies reveals oocytes throughout the whole year. Distinctly, each autozooid contains genital products of two size classes, *i.e.,* small oocytes arranged among larger ones (Fig. 4). Figure 5 presents size ranges of these two cohorts throughout the study. In the shallow reef small oocytes (<80 μm) appeared in September–October (Fig. 5a). In the deep reef the oogenic cycle started a bit earlier in August–September (Fig. 5b). Oocyte growth proceeded through the following 22–23 months. Mature oocytes with a diameter of 500–650 μm were recorded in July. In the deep reef ripe oocytes were also present later in August (Fig. 5b).

Spawning and development

The sequential monthly sampling of *S. glaucum* for about 4 years (Figs. 3, 5) indicated the approximate time of spawning, based on presence of oocytes or sperm sacs in a given month and their absence in the next. In 1980 we directly observed

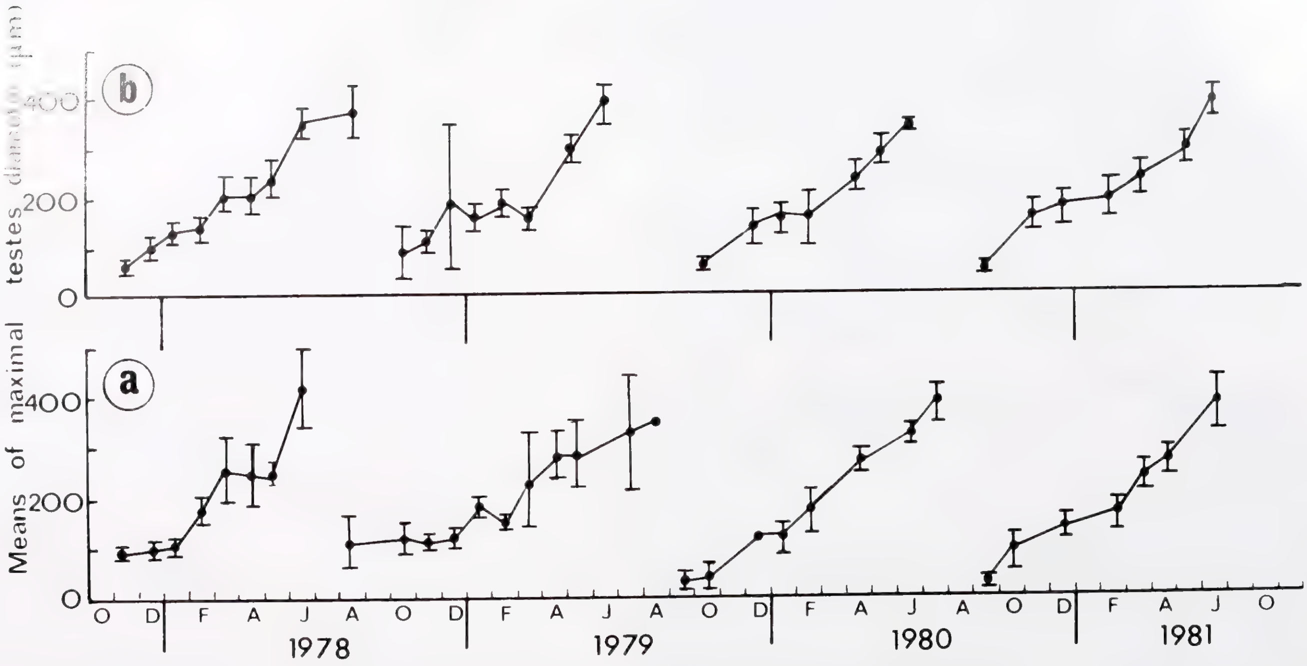

FIGURE 3. Monthly changes of the means of maximal sperm sacs diameter of *Sarcophyton glaucum* in 3–5 m (a) and in 27–30 m (b).

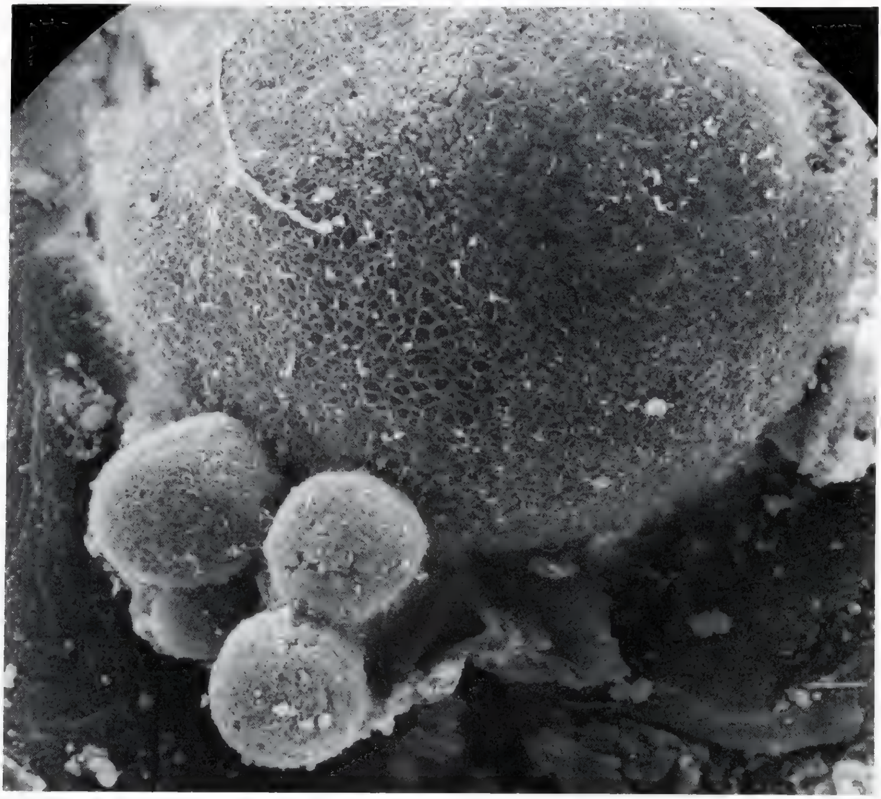

FIGURE 4. Two size classes of oocytes inside a polyp cavity of *Sarcophyton glaucum* (×220).

spawning of colonies maintained in aquaria. *S. glaucum* spawned during a single night (9 July), between 2000 and 2200 h. The eggs (Fig. 6a) are positively buoyant. A day later no genital products were observed in the polyp cavities of these colonies. Daily underwater surveys along a wide depth gradient (0–35 m) conducted prior to and on 9 July, indicated oocytes or testes in the entire population. Eighteen hours later (10 July) no oocytes or testes were found in colonies at a depth of 0–18 m. At greater depths, where only sparse populations of *S. glaucum* exist, few colonies still contained genital products, indicating a possibility of delayed spawning in this zone (see also Benayahu and Loya, 1983).

Colonies of *S. glaucum* were never found with embryos. Fertilization is probably external and 12 h after spawning blastulae were observed in the lab. A day later the majority of the embryos developed into pear-shaped young planulae. Further development, 36 h after fertilization, leads to the formation of a fully developed pink ciliated planula (Fig. 6b) measuring 900 μm in length and 250 μm in width. The larvae actively swam through the water, exhibiting various patterns of rotation or spiral swimming. They remained active for up to 14 days after spawning. Then they settled down on calcareous substrates and started to metamorphose into polyps.

DISCUSSION

The soft coral *S. glaucum* is a broadcasting species with externally developing larva. Gonadal structures of *S. glaucum* is similar to that of other soft corals (Benayahu

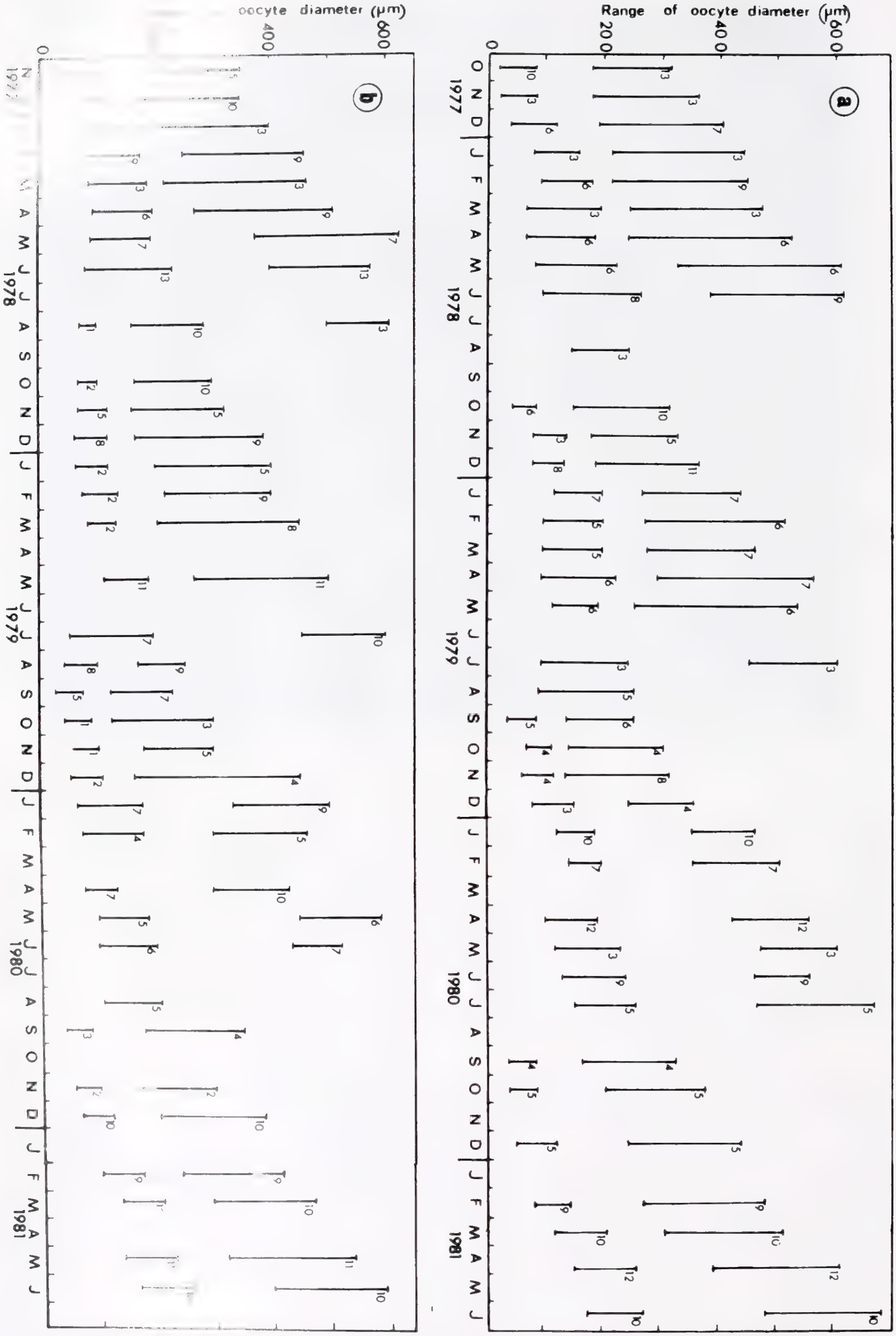

FIGURE 5. Monthly changes of the size range of oocyte cohorts of *Sarcophyton glaucum* in 3–5 m (a) and in 27–30 m (b). Sample size is indicated beside the bars (*i.e.*, number of female colonies examined in each date).

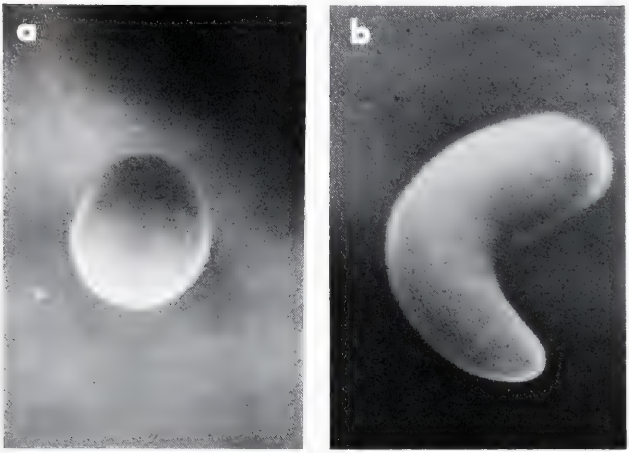

FIGURE 6. *Sarcophyton glaucum* a. Egg (×35). b. Mature planula (×50).

and Loya, 1984a). The gonads are borne only in the autozooids as in the majority of dimorphic alcyonaceans (Gohar, 1940; Yamazato *et al.* 1981). Previous evidence on age of onset of reproduction among soft corals is concerned with *Xenia macrospiculata* (2 years) and *P. f. fulvum* (3–4 years) (Benayahu and Loya, 1983, 1984a). The present study indicates that *S. glaucum* starts to reproduce only after gaining a substantially large colony size corresponding to 6–7 years of age for males, and 10 years for females (Benayahu, 1982). That is, young colonies of *S. glaucum* allocate energy for growth rather than for reproduction (see also van Moorsel, 1983). This feature enables the coral to initially capture space on the reef, and only at a later age to reproduce (Kojis and Quinn, 1981). Growth rate of *S. glaucum* is very slow (increase of disk diameter of only a few mm per year, Benayahu, 1982). Hence the large size of numerous *Sarcophyton* colonies, which have a disk diameter of 70–80 cm, suggests the existence of a long life span in this species.

Several studies on reproduction of stony corals present the maximal egg diameter of many species and discuss the biological significance of this parameter (Rinkevich and Loya, 1979; Fadlallah and Pearse, 1982; Harriot, 1983a, b). Only few comparative data are available on maximal oocyte diameter of soft corals. Table I shows the maximal egg diameter in several alcyonaceans and their mode of reproduction. Only some of these values are derived from long-term and sequential sampling of the species. Although the information at hand is incomplete, it appears that soft corals have large eggs (450–750 μm), regardless of their mode of reproduction. These findings do not coincide with the hypothesis made for stony corals suggesting that broadcasting corals might be expected to produce large eggs, whereas planulating species produce small eggs (Rinkevich and Loya, 1979; Fadlallah, 1983). Additional measurements of alcyonacean eggs (Benayahu, in prep.) together with the results presented in Table I suggest that large eggs are widespread among soft corals. Further studies are required in order to ascertain the significance of this conclusion.

A prolonged period of egg maturation as exhibited by *S. glaucum* (Fig. 5) was also recorded in *L. crassum* (Yamazato *et al.* 1981) and in two scleractinian corals (Fadlallah and Pearse, 1982). Completion of an oogenic cycle within 2 years leads to a simultaneous presence of two oocyte group sizes within a colony. Fadlallah and Pearse (1982) suggest that development of large oocytes requires such a long period of oo-

TABLE I

Maximal egg diameter in several soft corals and their mode of reproduction.

Coral species	Egg diameter (μm)	Mode of reproduction	Reference
Alcyonium digitatum (Linnaeus)	600	Broadcast	Hartnoll, 1977
A. hibernicum (Renouf)	500–640	Brood	Hartnoll, 1977
A. siderium Verrill	700	Brood	Feldman, 1970 (In: Sebens, 1983)
Lobophytum crassum von Marenzeller	600	Broadcast	Yamazato *et al.* 1983
Parerythropodium fulvum fulvum (Forskål)	600–700	Surface brooding	Benayahu and Loya, 1983
Sarcophyton glaucum (Quoy & Gaimard)	600	Broadcast	This study
Heteroxenia fuscescens (Ehrenberg)	700	Brood	Personal observation
Xenia macrospiculata Gohar	500	Brood	Benayahu and Loya, 1984a
X. umbellata (Savigny)	500–600	Brood	Ashworth, 1899
**Cladiella pachyclados* (Klunzinger)	450	—	Pratt, 1903
**Sinularia dura* (Pratt)	500	—	Pratt, 1903
**Anthelia formosa* Utinomi	450–700	—	Utinomi, 1950
**Heteroxenia elizabethae* (Kölliker)	750	—	Hickson, 1931

A (—) indicates that information was not supplied by the authors. An (*) indicates that egg size was derived from occasional measurements.

genesis. However, large oocytes (500–750 μm) in other soft corals achieve maturity within an annual cycle of less than a year (Benayahu an Loya, 1983, 1984a). We therefore conclude that large egg size *per se* is not the ultimate cause for a two year period of development. Species with a prolonged period of oocyte growth have a costly reproductive investment (Bell, 1980). We further suggest that a massive egg production coupled with synchronous maturation and very brief spawning period are characteristic of such species, thus demanding a long time for egg maturation. The slow growth rate of *S. glaucum* (Benayahu, 1982) provides an additional indication that its egg production leads to high energy expenditure over a long period of time (Fig. 5).

Recent studies on reproduction of scleractinian corals have demonstrated a large proportion of broadcasting species (Kojis and Quinn, 1982; Harriot, 1983a; Babcock, 1984; Harrison *et al.* 1984; Shlesinger and Loya, 1985). Currently, among reef soft-corals, only two species are known to spawn pelagic gametes (Table I: *L. crassum* and *S. glaucum*, see also Bowden *et al.* 1985). A brief period of spawning each year occurs in many scleractinian corals (Harrison *et al.* 1984, Shlesinger and Loya, 1985). In contrast, *S. glaucum* is the only soft coral thus far documented with such a mode of gamete release. Undoubtedly the extent of broadcasting in this group has to be adequately determined.

S. glaucum has a markedly short period of planular development (36 h). Previous studies indicate that oviparous octocorals have a rather short embryogenesis: *A. digitatum*, 3–4 days (Matthews, 1917); *Renilla köllikeri*, 2 days (Satterlie and Case, 1979); *Clavularia obesa*, 2 days (Mori and Tanase, 1973); *Pistilosarcus gauernyi*, 3–4 days (Chia and Crawford, 1973). Information on duration of planular embryogenesis in brooding octocorals includes the alcyonaceans *Xenia macrospiculata*, 14 days (Benayahu, 1982) and *Parerythropodium fulvum fulvum*, 6 days (Benayahu and Loya, 1983). These findings may imply a rapid planular development among broadcasting octocorals, whereas brooding in this group involves a prolonged embryogenesis. Spawned gametes are exposed to dangers such as predation or mechanical damage. We therefore suggest that in broadcasting octocoral species, faster embryogenesis increases spat survival and the chances for successful colony development. Octocoral larvae are sluggish and spend only a few hours in the water column prior to their final attachment (Benayahu and Loya, 1983, 1984b). Conversely, planulae of the broadcasting octocoral *S. glaucum* are capable of swimming far away from their parents hence promoting wide dispersal and extensive geographical distribution (Verseveldt, 1982).

Life history traits of *S. glaucum* are comprised of large body size, late age at the onset of reproduction, slow growth rate, and a long life span. Its reproductive strategy demonstrates a prolonged oogenic cycle coupled with a brief annual spawning. The pelagic eggs and planulae are exposed to a high mortality rate, hence offspring survival is decreased. In addition, *S. glaucum* has stable population size and poor colonization capabilities of newly exposed reef surfaces (Benayahu, 1982). In view of these life history traits, *S. glaucum* may be regarded as a K-selected species (*sensu* Pianka, 1970). This soft coral has never been observed to be excluded by other benthic reef organisms nor suffer from predation (see Ne'eman *et al.,* 1974). Therefore, even if a broader aspect of K-selection is considered including interspecific competitive ability and predator avoidance (Green, 1980), *S. glaucum* may be further viewed as a K-selected species. We have elsewhere demonstrated that life history characteristics of the soft coral *X. macrospiculata* include small body size, early onset of reproduction, high fecundity, prolonged planulation period and capacity to rapidly exploit space all year round (Benayahu and Loya, 1984a, b, 1985). Therefore, the two soft corals *X. macrospiculata* and *S. glaucum* demonstrate markedly different life history tactics and may be situated on opposing ends of an "r-K continuum" (*sensu* Pianka, 1970).

ACKNOWLEDGMENTS

We express our gratitude to the late Prof. Ch. Lewinsohn (Tel Aviv University) for his helpful advice during the study. We thank N. Holland and D. Dexter for critically reviewing the manuscript and offering valuable comments. We thank the M.B.L. staff at Eilat for their hospitality and facilities and L. Maman and A. Shoob for taking the photographs.

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INHIBITION OF GERMINAL VESICLE BREAKDOWN AND ACTIVATION OF CYTOPLASMIC CONTRACTILITY IN *SPISULA* OOCYTES BY CALMODULIN ANTAGONISTS

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ABSTRACT

We have treated *Spisula* oocytes with the calmodulin antagonists, chlorpromazine, calmidazolium, and W-7, and the inactive W-7 analog, W-5, to examine the role of calmodulin in meiosis reinitiation and egg activation in this species. Chlorpromazine and W-7 both inhibited germinal vesicle breakdown (GVBD) at 50–75 μM, whether triggered parthenogenetically by KCl activation or by fertilization. Calmidazolium was effective at *ca.* 3 μM. W-5 was ineffective. However, none of the anticalmodulin drugs inhibited GVBD when induced by ionophore A23187. Time course studies showed that W-7 was effective at inhibiting KCl-induced GVBD only when added within 1 min after KCl. Similar studies using the calcium channel blocker, verapamil, showed that the period during which calcium channels are required for GVBD substantially exceeds the interval of sensitivity to W-7. Calmodulin thus acts at a stage prior to the calcium flux known to be required for GVBD.

Chlorpromazine, calmidazolium, and W-7 triggered ameboid contractions of the immature oocyte in addition to inhibiting GVBD. W-5 was again ineffective. The ameboid contractions observed are not typical of parthenogenetically activated oocytes of this species. Experiments in which chlorpromazine-treated oocytes were simultaneously treated with low concentrations of cytochalasin B showed that the cytoplasmic contractions are microfilament-dependent. The results indicate that the contractions triggered by the calmodulin antagonists are not a part of the normal egg activation process and that cytoplasmic contractions can be induced in this species independently of egg activation and may result from perturbation of a calmodulin-microfilament interaction.

INTRODUCTION

Calmodulin has been implicated in the regulation of germinal vesicle breakdown (GVBD) in several species, including starfish (Meijer and Guerrier, 1981; Dorée *et al.,* 1982), frogs (Wasserman and Smith, 1981), annelids (Carroll and Eckberg, 1983), and mice (Bornslaeger *et al.,* 1984). The involvement of calmodulin in the regulation of GVBD may be general and, therefore, basic to a fundamental intracellular mechanism regulating the process of GVBD, because the apparent "triggering" event for GVBD is not the same for all these forms. Starfish and vertebrates undergo GVBD in response to hormonal stimulation, whereas the annelid, *Chaetopterus,* undergoes GVBD upon contact of the oocytes with sea water.

Additionally, calmodulin has been implicated in the control of egg activation in sea urchins (Baker and Whitaker, 1980; Steinhardt and Alderton, 1982) and annelids

Received 12 August 1985; accepted 31 October 1985.

(Carroll and Eckberg, 1983). In sea urchins, the criterion for egg activation has been cortical vesicle exocytosis, whereas in the annelid, *Chaetopterus,* the criterion for activation was the onset of ameboid contractions characteristic of the early phases of differentiation without cleavage, a usual result of parthenogenetic activation in this species (Lillie, 1902; Eckberg, 1981). In our initial study, treatment with calmodulin antagonists resulted in ameboid contractions more violent than usually observed during differentiation without cleavage.

The present investigation further examined the possible involvement of calmodulin in the regulation of germinal vesicle breakdown by analysis of an organism in which this process is normally triggered by a third mechanism, fertilization, and compared any calmodulin involvement in fertilization-induced GVBD with parthenogenetically initiated GVBD in the same species. The results indicate that calmodulin is involved in GVBD in this species, as well. Analysis of the kinetics of appearance of the inhibitory effect indicates that calmodulin participates in early events of maturation and activation in this species. Studies involving ionophore activation and the calcium channel blocker, verapamil, show that a calcium flux and a calcium-dependent step that is independent of calmodulin follows the calmodulin-dependent period. Additionally, antagonist-induced ameboid contractions were evidently independent of normal egg activation and resulted directly or indirectly from an effect on a microfilament-dependent cytoplasmic contractile system.

MATERIALS AND METHODS

Mature specimens of *Spisula solidissima* were obtained from the Marine Resources Division, Marine Biological Laboratory, and handled by standard methods (Costello and Henley, 1971). Oocytes were washed 3–4 times with seawater prior to use. Batches of oocytes were not used unless >95% had intact germinal vesicles prior to treatment and >95% underwent GVBD upon treatment with 6% 2.5 *M* KCl (Allen, 1953).

All antagonists and ionophore A23187 were obtained from Sigma Chemical Co. Chlorpromazine, verapamil, calmidazolium, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W-7), and N-(6-aminohexyl)-1-napthalenesulfonamide (W-5) were made fresh before each experiment as a 10 m*M* solution in ethanol or dimethyl sulfoxide. Ionophore A23187 and cytochalasin B were stored as stock solutions at −20°C. Ionophore A23187 was made as a 2.5 m*M* stock in dimethyl sulfoxide (DMSO). Cytochalasin B was made as a 1 mg/ml stock in DMSO. Calmodulin antgonists were added to oocytes at the concentrations given in the figure legends. Ionophore A23187 was added to oocytes at a final concentration of 2.5 μM. This concentration is effective at initiating GVBD in this species (Schuetz, 1975; Eckberg, 1983). Cytochalasin B was added at various concentrations ranging from 0.03 to 3 μg/ml. Control experiments were always performed in which either DMSO or ethanol was added without antagonist.

Artificial seawater (ASW), calcium-free artificial seawater (CFSW), and calcium- and magnesium-free artificial seawater (CMFSW) were made according to standard MBL formulae (Cavanaugh, 1964). All artificial seawaters contained 10 m*M* TRIS-HCl, pH 8.2, to prevent possible pH-dependent nonspecific effects of the acidic antagonists (Allen, 1953; Carroll and Eckberg, 1983).

When GVBD was induced by KCl or ionophore, oocytes were usually exposed to the antagonist for 2 min prior to addition of KCl. In experiments in which GVBD was induced by fertilization, oocytes were inseminated with sufficient sperm to achieve synchronous fertilization, and antagonists were added 1 min later. Oocytes and embryos were examined by phase-contrast and Nomarski differential interference contrast microscopy at intervals for at least one hour after treatment for evidence of GVBD, ameboid contractions, and any other changes.

RESULTS

Calmodulin antagonists can block GVBD

Since KCl induces GVBD rapidly and synchronously in oocytes in this species, and KCl-induced GVBD would be independent of possible indirect inhibition through inhibition of fertilization, we initially tested the ability of calmodulin antagonists to prevent GVBD on KCl-activated oocytes. Figure 1 shows that all calmodulin antagonists tested blocked GVBD in *Spisula* oocytes when induced by KCl. The results also show that calmidazolium is maximally effective at *ca.* 3 μM, a substantially lower effective concentration than observed for chlorpromazine or W-7 (*ca.* 50–75 μM), and that W-5 is ineffective at concentrations up to 200 μM.

We also tested the effects of calmodulin antagonists on GVBD when induced by fertilization, the natural trigger in this species. Figure 2 shows that all calmodulin antagonists tested inhibited GVBD when induced by fertilization. Furthermore, the effective concentrations of the antagonists for fertilization-induced GVBD were similar to those for KCl-induced GVBD. Calmidazolium was maximally effective at *ca.* 3–10 μM, chlorpromazine and W-7 were maximally effective at *ca.* 75 μM, and W-5 was ineffective at any concentration up to 200 μM. The antagonists had no effect on post-GVBD development through first cleavage, because oocytes which successfully underwent GVBD in the presence of the inhibitors after fertilization also formed polar bodies and cleaved normally and synchronously with controls.

To determine the period of sensitivity to calmodulin antagonists for inhibition of GVBD, KCl-stimulated oocytes were exposed to 100 μM W-7 at various times after KCl stimulation and examined for GVBD 20 min later. Figure 3 shows that GVBD was prevented only if W-7 was added within 1 min after KCl addition. Similar results were obtained using calmidazolium (data not shown).

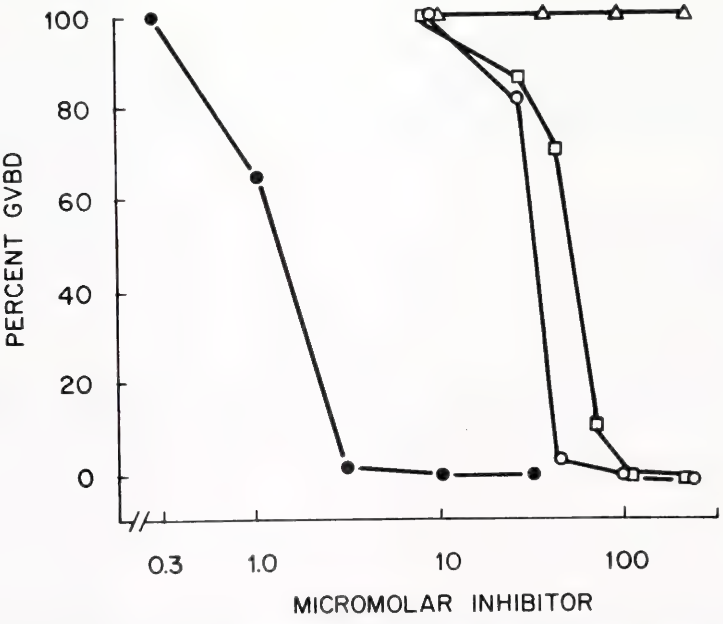

FIGURE 1. Effect of calmodulin antgonists on KCl-induced GVBD in *Spisula* oocytes. Oocytes were treated with different concentrations of calmidazolium (filled circles), chlorpromazine (open circles), W-7 (squares), or W-5 (triangles). Two minutes later they were exposed to 6% 2.5 *M* KCl and examined for GVBD 20 min later.

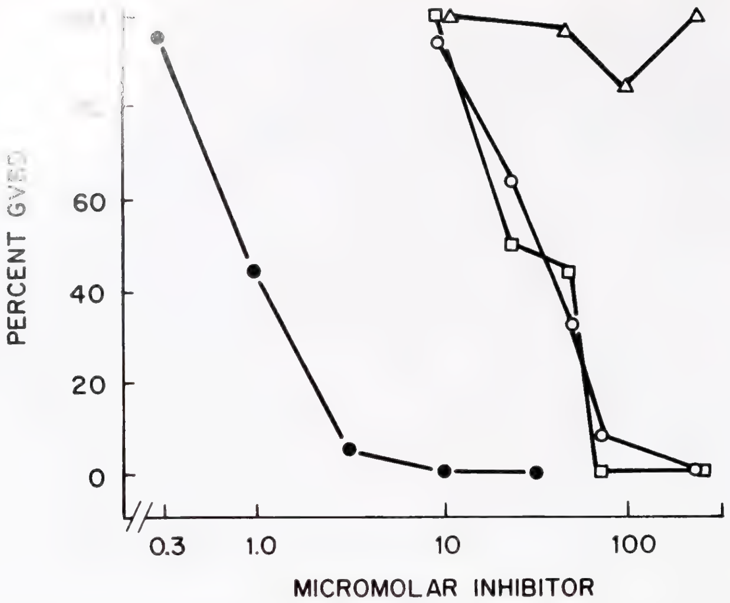

FIGURE 2. Effect of calmodulin antagonists on fertilization-induced GVBD in *Spisula* oocytes. Oocytes were inseminated, and 1 min later they were exposed to different concentrations of calmidazolium (filled circles), chlorpromazine (open circles), W-7 (squares), or W-5 (triangles). They were examined for GVBD 20 min later.

Calmodulin antagonists do not prevent ionophore activation of Spisula *oocytes*

Ionophore A23187 activation of *Spisula* oocytes apparently involves a different pathway than activation by KCl or fertilization (Eckberg, 1983). Specifically, ionophore activation should result directly from initiation of a calcium ion flux. Accordingly, we tested the effects of calmodulin inhibitors on ionophore A23187-induced GVBD. Table I shows that none of the drugs inhibited ionophore-induced GVBD.

A continued calcium flux is required after the calmodulin-dependent steps

The timing of the effect of calmodulin antagonists and the lack of inhibition of ionophore-induced GVBD by calmodulin antagonists suggested that a continued calcium flux after the calmodulin-dependent step might be required for GVBD in this species. Since calcium channel blockers are known to prevent the required calcium flux and GVBD (Guerrier *et al.,* 1981), we treated oocytes with the calcium channel blocker, verapamil. Figure 3 shows that verapamil blocks GVBD when added up to 2½ minutes after KCl treatment. Therefore, the calcium channels are required for 1–2 min after the calmodulin antagonist-sensitive period.

Oocytes treated with calmodulin antagonists undergo ameboid contractions without GVBD

Oocytes exposed to ≥ 50 μM chlorpromazine or W-7 or to ≥ 3 μM calmidazolium often underwent violent ameboid contractions. The contractions observed included formation and retraction of pseudopod-like protrusions and often resulted in net movement of the oocytes across the substratum. Initiation of ameboid contractions

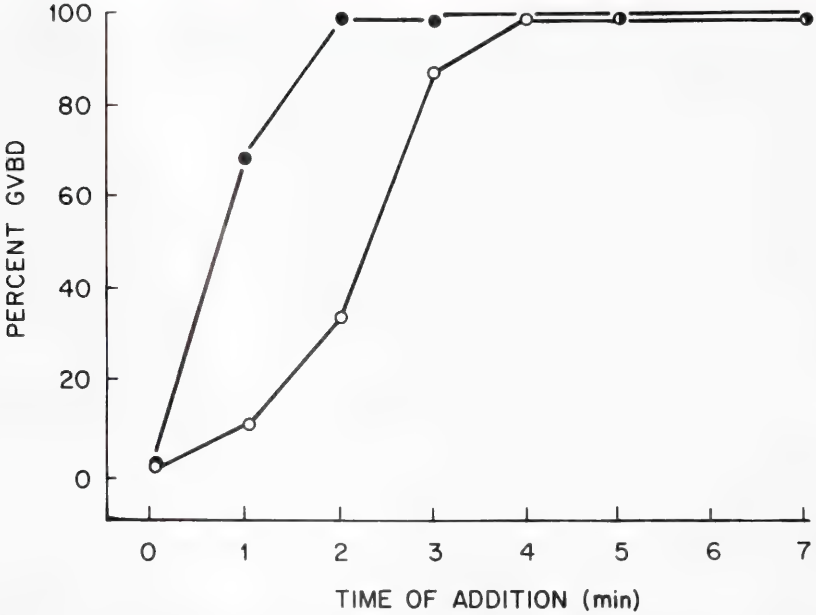

FIGURE 3. Time of sensitivity to W-7 and verapamil of KCl-induced GVBD in *Spisula* oocytes. Oocytes were exposed to 6% 2.5 M KCl at T = 0 min and 100 μM W-7 (filled circles) or 75 μM verapamil (open circles) was added at the times indicated. Oocytes were examined for GVBD 20 min after KCl addition. The times of addition at which GVBD was inhibited 50% were determined using curve-fitting software to be 0.94 ± 0.16 min for W-7 and 2.47 ± 0.69 min for verapamil. These times were significantly different from each other by the t-test ($P < 0.05$).

occurred in CFSW and CMFSW as well as in ASW and occurred whether or not oocytes had been previously inseminated or stimulated with KCl but only if the germinal vesicles had not broken down (data not shown). Examples of the kind of contractions and deformations observed are shown in Figure 4c, d, e. In many cases, these severe contractions obliterated the germinal vesicles (*cf.* Fig. 4e).

Observation of ameboid contractions suggested activation of a microfilament-dependent cytoplasmic contractile system. To test this, we simultaneously treated oocytes with 75 μM chlorpromazine and various concentrations of cytochalasin B (CB). As illustrated by Figure 4f, 0.3 μg/ml CB prevented the ameboid contractions. This treat-

TABLE I

Ineffectiveness of calmodulin antagonists at inhibition of GVBD induced by ionophore A23187

Antagonist[1]	% GVBD	±SD
none	98	2
calmidazolium	100	1
W-7	83	12
W-5	84	8
chlorpromazine	95	3
no ionophore	3	1

[1] Chlorpromazine, W-7, and W-5 were 100 μM; calmidazolium was 10 μM; ionophore A23187 was 2.5 μM. Three experiments were performed.

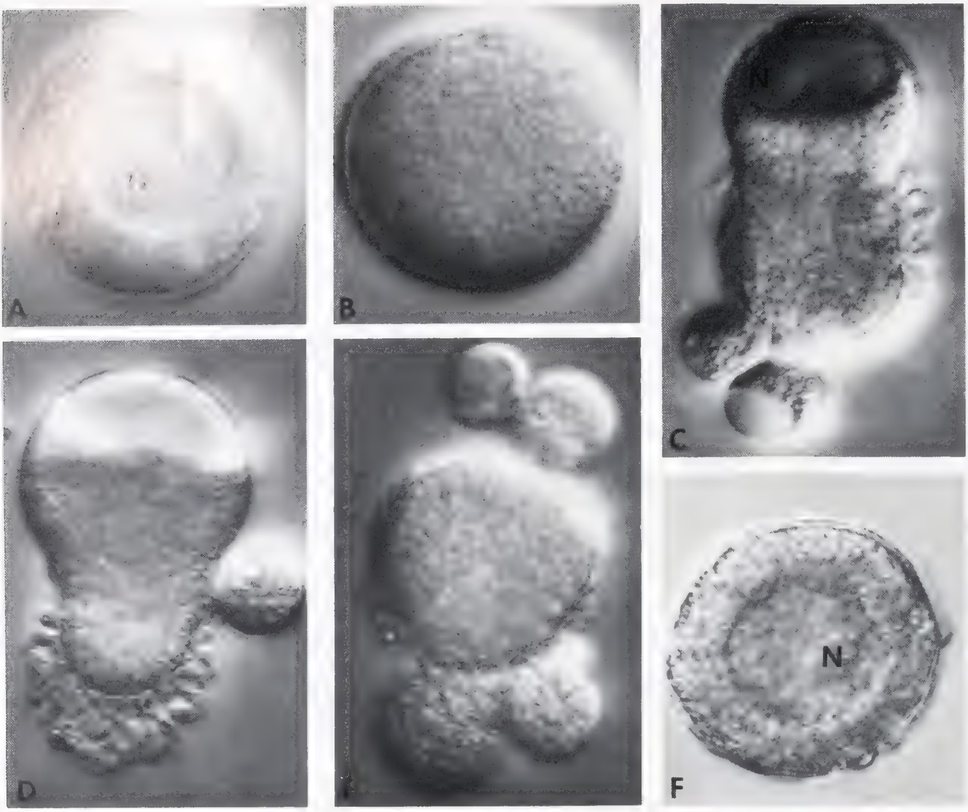

FIGURE 4. Ameboid contractions induced in *Spisula* oocytes by treatment with 75 μM chlorpromazine. A. Primary oocyte prior to treatment. B. KCl-stimulated oocyte that has undergone GVBD. C., D., E. Examples of oocytes showing severe ameboid contractions after treatment with 75 μM chlorpromazine. Note that in C the germinal vesicle is eccentric and contains a nucleolus, in D the eccentric remnant of the nucleolus lacks a nucleolus, and in E the germinal vesicle has been obliterated. This type of behavior is typical. F. An oocyte treated with 75 μM chlorpromazine and 0.3 μg/ml cytochalasin B. The ameboid contractions have been prevented. N = nucleolus.

ment blocked contractions when CB was added simultaneously with the calmodulin antagonist and stopped the contractions when added after they had begun. CB treatment also showed that the apparent obliteration of the germinal vesicles in ameboid oocytes (Fig. 4e) also resulted from the contractile activity. When the ameboid contractions were prevented, the germinal vesicles persisted (Fig. 4f).

DISCUSSION

The results of this study provide important new evidence concerning the involvement of calcium and calmodulin in germinal vesicle breakdown. Calmodulin involvement is demonstrated by the fact that several structurally different calmodulin antagonists block GVBD at concentrations similar to those at which they are known to affect calmodulin (Hidaka *et al.*, 1980; Weiss and Wallace, 1980), and that an analog of one of the antagonists which has little anticalmodulin activity has no effect on GVBD. Calmodulin had been implicated previously in GVBD in starfish (Meijer and Guerrier, 1981), amphibians (Wasserman and Smith, 1981), and the mouse (Bornslaeger *et al.*, 1984), which normally undergo GVBD in response to hormonal stimulation and the annelid, *Chaetopterus,* (Carroll and Eckberg, 1983) which normally undergoes GVBD upon contact of the oocyte with seawater. *Spisula* normally undergoes GVBD in response to fertilization, and can also undergo GVBD in response to parthenogenetic stimulation (Allen, 1953; Schuetz, 1975). The results of this study demonstrate that, in this case as well, GVBD is a calmodulin-dependent event. Taken together with previous results, these studies strongly suggest that calmodulin involvement in GVBD is a general and therefore probably fundamental phenomenon.

The present results demonstrate that calmodulin is active at an early stage in GVBD, because addition of W-7 as little as 1 min after KCl activation was ineffective at inhibiting GVBD. Dorée *et al.* (1982) suggested that calmodulin acts at the oocyte surface and is involved in the transduction of the hormonal message in the initiation of GVBD in starfish because injected anticalmodulin drugs and antibodies were ineffective and inhibition of GVBD by external anticalmodulin drugs could be overcome by increasing the hormone concentration. The results of our experiments are consistent with a calmodulin effect at the cell surface because of the rapidity of GVBD inhibition by externally applied calmodulin antagonists. The results of the present study and our previous study on *Chaetopterus* extend the hypothesis of Dorée *et al.* to species in which GVBD is not hormone-dependent.

The results of the ionophore and verapamil studies provide additional information about the roles of calcium and calmodulin in the activation of the *Spisula* oocyte. None of the calmodulin antagonists blocked ionophore-induced GVBD, indicating that the calmodulin-dependent steps occur prior to the calcium flux. This also shows that the effects of the calmodulin antagonists in inhibiting GVBD do not result from generalized toxicity. Treatment with verapamil indicated that calcium channels must be open for 2–3 minutes for GVBD to occur. This period substantially exceeds the period of sensitivity to calmodulin antagonists. Together, these studies show that while calmodulin is required for GVBD, there is at least one additional calcium-sensitive event subsequent to the calmodulin-dependent step involved in the initiation of GVBD. These results imply that calmodulin is involved in the initiation of the required calcium uptake. Calmodulin has also been implicated in the regulation of calcium transport in erythrocytes (Muallem and Karlish, 1980; Larsen *et al.,* 1981).

Our experiments suggest the following model for activation. Sperm or a parthenogenetic agent contacts the surface of the oocyte, and initiates a reaction or series of reactions that involve calmodulin at an early step. These reactions, in turn, result in an increase in intracellular calcium, probably by uptake from the surrounding seawater (Guerrier *et al.,* 1981; Jaffe, 1985). This increase in intracellular calcium triggers the events that result in GVBD and polar body formation.

The activation of ameboid contractions by calmodulin antagonists had been reported previously in *Chaetopterus* oocytes (Carroll and Eckberg, 1983). In that species, ameboid contractions are a normal result of parthenogenetic activation (Lillie, 1902; Eckberg, 1981) and could therefore represent normal egg activation. In *Spisula,* however, ameboid contractions are not normally associated with egg activation, although several kinds of cytoplasmic movements have been reported to occur under certain conditions (Rebhun, 1963). The contractions reported here differ from those previously identified by Rebhun. The contractions observed in this study occurred in oocytes which had not undergone GVBD, whereas those in Rebhun's study took place only in eggs which had undergone GVBD.

While the violent ameboid contractions we observed sometimes resulted in cytolysis, they were not simply the result of cytolysis because when oocytes were treated with cytochalasin B simultaneously with the calmodulin antagonists, the contractions were blocked. Additionally, when CB was added to oocytes undergoing these contractions, they relaxed to a spherical shape. These contractions, therefore, represent a cytoplasmic contractile activity that is microfilament-dependent and directly or indirectly initiated by calmodulin antagonists. Calmodulin antagonists have been shown previously to disrupt cytoskeletal organization in mammalian (Osborn and Weber, 1980) cells as well as sea urchin eggs (Carroll and Eckberg, unpub.) and embryos (Hagström and Lönning, 1973) and *Chaetopterus* oocytes (Carroll and Eckberg, 1983). The mechanism by which calmodulin antagonists initiate these contractions is unknown and currently under study.

ACKNOWLEDGMENTS

This research was supported in part by NIH HD18255 to W.R.E. and by a Purdue XL grant to A.G.C.

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DO SYMBIOTIC PEA CRABS DECREASE GROWTH RATE IN MUSSELS?

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ABSTRACT

Pea crabs living within the mantle cavities of a variety of bivalve hosts have several adverse effects. In blue mussels (*Mytilus edulis* L.), the crab *Pinnotheres maculatus* (Say) steals food strands and causes gill lesions. We studied the long-term stress of *P. maculatus* on its host by measuring shell accretion in the field, and by numerically characterizing shell shape. Shell form in *M. edulis* is presumed to reflect environmental and physiological history. We computed growth increments in infested populations of mussels over a three-month period at two sites with high and low nutrient regimes. When growth was measured by change in shell length, significant differences between mussels with and without large pea crabs occurred at the low nutrient site, but not where mussels enjoyed a high nutrient regime. To integrate very long-term disparity in growth rates associated with infestation, we used mussels from a robust, naturally occurring population. We recorded and analyzed mussel silhouette shapes with a video digitizer. Elliptic Fourier approximation completely characterized the two-dimensional outlines of shells in such a way that the allometric dependence of shape variables on shell size could be easily removed. At this evidently favorable site, infested mussels displayed significant shell shape distortion characteristic of reduced growth rates. Thus, even in apparently benign environments, pea crab infestation appears to be a chronic stress to *M. edulis.*

INTRODUCTION

Pinnotherids, or pea crabs, are a group of decapod crustaceans adapted for life within other marine animals. Two hundred fifty-two species of pinnotherids have been described (Schmitt *et al.,* 1973). In this spectrum of species, there is a gradation from specialist crabs to generalist crabs with regard to host and geographic range. *Pinnotheres maculatus,* the species in this study, can be considered an ultimate generalist, "demonstrating a profound lack of host specificity" (McDermott, 1962). It has a host list of at least 21 species in several bivalve and polychaete families, and is distributed throughout the subarctic, temperate and tropical zones.

Females of *Pinnotheres maculatus* are soft-shelled and live their entire adult lives within the host—in this case the bivalve *Mytilus edulis.* The crab positions itself on the gills and uses its chelae to pick up mucous food strands from the host that pass by. This species of pea crab has "dwarf" males; they rarely exceed 6 mm across the carapace, and are able to move freely from mussel to mussel. Males can feed independently of mussels (Caine, 1975) and therefore may not represent as much of a drain on a host as a female. Small, immature female pea crabs are also able to move from host to host. When a female pea crab is larger than approximately 6 mm across the carapace, it is unable to leave its host easily because the mussel's gape is not large

Received 25 March 1985; accepted 16 October 1985.

enough. *P. maculatus* females do not produce eggs until the summer after they reach the first true-crab stage (Pearce, 1964). Since the smallest ovigerous females are 6 mm across the carapace, pea crabs of this size must be one or more years old (*cf.* growth curves for *P. ostreum* in Christensen and McDermott, 1958). Large pea crabs have occasionally remained within their hosts for at least one growing season.

While pea crabs have experimentally detectable effects on several variables measured for bivalve hosts (Haven, 1959; Silas and Alagarswami, 1965; Pearce, 1966; Kruczynski, 1972; Anderson, 1975; Pregenzer, 1979, 1981), their long-term cumulative effect on host physiology is unknown. Pea crabs can live in a host for at least two to three years (Pearce, 1966; manuscript by N. Bourne, Department of Fisheries and Oceans, Pacific Biological Station, Canada, "Pinnotherid crabs in butter clams, *Saxidomus giganteus:* a commensal or parasite"). The energetic demand of supporting a pea crab may impair host response to long or short term stresses, and might therefore be reflected in a variety of parameters.

If the long-term negative effects of pea crab infestation are to be characterized, an integrated record of the host's physiological history is needed. The bivalve shell provides such a record. Rhoads and Pannella (1970) and Pannella and McClintock (1968) demonstrated that "microgrowth" changes due to varying food, temperature, *etc.,* are reflected in the molluscan shell. Seed (1968) and Lewis and Powell (1961) showed that differences in mussel shell shapes vary with age but also with the environmental conditions to which the animals had been subjected. The degree of divergence between the shapes of two mussels of the same age should be related to the time spent under differing conditions. In general, a fast-growing mussel is triangular or "spatulate" because large, even shell increments are added to the posterior of the shell. Slow-growing mussels add smaller and less even shell increments; over time, the shell therefore takes on an elongate and slightly recurved or "ungulate" appearance. These two forms are illustrated in Figure 1A.

We examined the effects of the generalist crab *Pinnotheres maculatus* on shell growth of its bivalve host *Mytilus edulis.* We measured differences in growth rate over the short term with field experiments, and over the long term by an analysis of shell shape using an elliptic Fourier technique (*cf.,* Gevirtz, 1976).

MATERIALS AND METHODS

Mussels were collected from a depth of 30 meters off Gay Head, Martha's Vineyard, Massachusetts. To avoid using very young or very old mussels, only those in the length

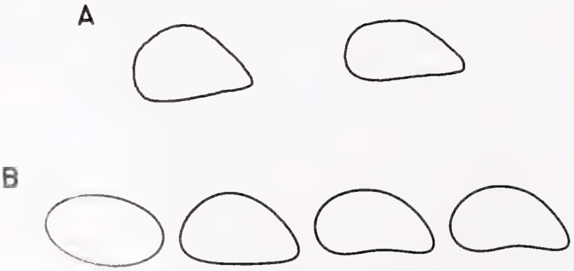

FIGURE 1. Mussel shell shape and its elliptic Fourier decomposition. A. At left, a triangular or "spatulate" mussel shell form characteristic of fast-growing mussels. At right, an elongate and slightly recurved "ungulate" form characteristic of slowly growing animals. B. Successively better approximations of a typical mytilid contour using 1, 2, 3, and then 4 harmonics from an elliptic Fourier decomposition of shell outline.

range of 50.0 to 90.0 mm were used for experimentation. This selection avoids the predictable and extreme effects on shape due to age. Very young animals are known to be spatulate while very old ones become ungulate (Lewis and Powell, 1961). Of mussels from this sample, 199 were dissected immediately to determine the percentage of infestation by pinnotherids.

Field experiment

One hundred twenty animals were engraved with an identification number and were measured to the nearest 0.1 mm with vernier calipers for length (maximum anterior-posterior axis), height (maximum dorso-ventral axis), and width (maximum lateral axis). These mussels were suspended in wide-mesh cages 2 meters above the substrate in two nutritionally different environments off Long Island, New York. These transplants suffered, on average, 12% mortality. After three months (June through August 1980), 100 surviving animals were re-measured and dissected, and the size and sex of each pea crab was recorded. We used analysis of variance to assess the effects of site and pea crab infestation on growth rate as measured by the three morphological dimensions of the host. Infestation levels before and after the experiment were compared using a G-test.

The 'nutrient-rich' environment was in protected harbors of Long Island Sound (Port Jefferson) and the 'nutrient-poor' environment was off the south shore of the island (Shinnecock). Previous work by independent researchers supports this characterization. Newell *et al.* (1982) showed the energy content of available seston averaged $50 \text{ J} \cdot \text{l}^{-1}$ near the first site *versus* $20 \text{ J} \cdot \text{l}^{-1}$ at the second during the summers of 1978 and 1979. Over the last ten years, the nutrient-rich environment has also been about three times higher in levels of phosphate and total inorganic nitrogen (nitrate, nitrite, and ammonia) than the nutrient-poor environment (data supplied by R. Nuzzi and M. Waters, Suffolk County Department of Health, pers. comm.).

Shape analysis

Eighty-two right valves of *Mytilus edulis* were used in a comparative shell shape analysis. These shells were collected from the same biological population at Gay Head, but were not used in the field experiment described above. Thus they form a statistically independent sample of mussels from that population. Using a video camera, we recorded the two-dimensional silhouette of each animal on a bright background. A computer program traced the outline of each valve, which is defined as the locus of points at which brightness changes from dark to light, or light to dark, on vertical or horizontal transects of the image. The details of the technique are discussed by Ferson *et al.* (1985).

We used elliptic Fourier decomposition (Kuhl and Giardina, 1982) to compute coefficients that describe shell shape. This is a very general method that, by treating x-coordinates independently of y-coordinates and by using only the first differences of change in either direction, asymptotically yields a perfect fit to practically any closed curve. We computed the Fourier coefficients for the first four harmonics for each shell. Since x- and y-directions are evaluated separately for sine and cosine terms, there are four coefficients per harmonic. However, three of the 16 coefficients degenerate under normalizations for trace starting point and shell position, size, and rotation. We therefore used 13 nontrivial coefficients. These were more than adequate to describe these simple forms. Figure 1B displays a sequence of improving approximations to a shell outline using 1, 2, 3, and then all 4 harmonics. The coefficients constitute a numerical decomposition of the shell's entire two-dimensional shape. We averaged these coefficients elementwise to obtain the mean shell form for each group (with and without large crabs). Following Younker and Ehrlich (1977), we used the coefficients as variables in a multivariate test of difference in central tendency between the groups. We used Mahalanobis' D^2 statistic (Mahalanobis, 1936) as a measure of this difference and examined its significance with a randomization test (Sokal and Rohlf, 1983, p. 787). Although the Fourier coefficients were arithmetically normalized for scale change (Kuhl and Giardina, 1982), we used a multivariate analysis of covariance with mussel length as the covariate to account statistically for any allometry in shell shape. For purposes of display we also computed the linear discriminant function separating the groups for these Fourier descriptors. This use of the discriminant function is a distribution-free technique (Lachenbruch, 1975).

RESULTS

In the dissected sample, 69% of the mussels contained a pea crab; 31% had females over 6.0 mm, and 38% had smaller females or males. Over the size range 50.0 to 90.0 mm in length, there is no significant correlation between the size of pea crab and the size of mussel host ($P > 0.2$; $R^2 = 0.04$). Figure 2 displays the scattergram.

Field experiment

Following the three-month field experiment, thirty-nine out of 100 surviving mussels harbored a pea crab of any size. Thirty-five (or 90%) of these had a mature female crab. Thus, the infestation level by small pea crabs, which had been 38% in the immediately dissected sample, dropped to 4% over three months in a population of suspended hosts. The difference between these frequencies is significant at the $P < 0.001$ level by a goodness of fit G-test using Williams' correction (Sokal and Rohlf, 1983, p. 704). Since crabs can live two to three years (Christensen and McDermott, 1958), normal mortality does not account for this reduction. However, since crabs attempting to change hosts would likely be lost from the sample by falling to the substrate, a high frequency of host-switching would account for reduced infestation by small crabs. In any case, one can infer that only large female pea crabs remain within their hosts over long periods of time.

Using original shell length as the covariate, we did a multivariate analysis of covariance on measured increments for the three shell dimensions after the short-term growth experiment. The average values are displayed in Table I. The mancova detected no significant differences in growth increment across the size range of mussels used. Thus the narrowness of this range seems to have controlled for allometric dependence of growth on size. Under Wilk's criterion (Rao, 1973, p. 555), we observed a highly significant effect due to site (F = 6.59; $P < 0.0005$). The mussels growing in the environment that we had designated as 'high-nutrient' grew substantially more, especially in the length dimension, than mussels growing in the 'low-nutrient' environment. We take this result as biological confirmation of the expectation based on chemical and energetic analyses of nutrient levels in these environments.

The analysis also detected a significant effect on growth increment due to infestation by large pea crabs (F = 2.91; $P < 0.038$). Since the bulk of this effect was contained in the length increment, we did a (univariate) two-way analysis of variance to explore this difference. In this closer look, however, we observed an interesting and significant interaction between pea crab infestation and site effects. In particular, there was little change in mussel growth increment due to pea crabs at the high-nutrient environment,

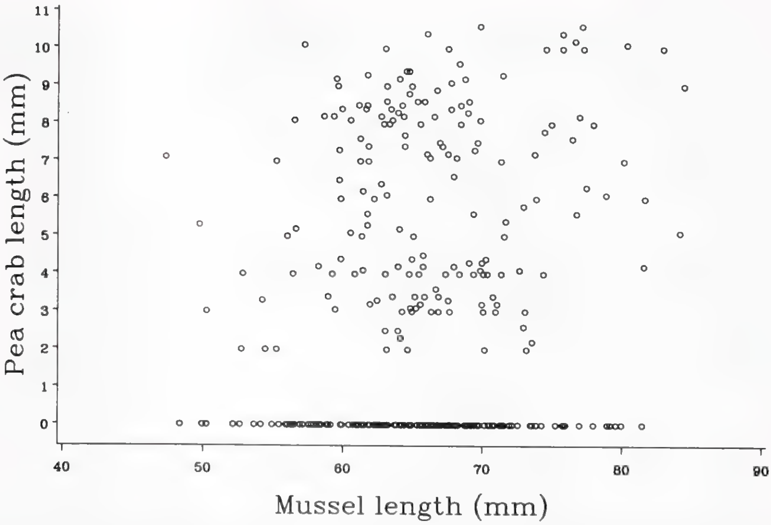

FIGURE 2. Symbiont pea crab carapace length as a function of host mussel shell length. This relation seems to be governed only by gross physical constraints on the largest crab a mussel of a given size can harbor. For the mussel size range of 50 to 90 mm, the regression is not statistically significant.

while at the nutrient-poor environment, the presence of an adult female crab ($\geqslant 6.0$ mm) resulted in a significantly lower growth increment in length ($P < 0.01$). Figure 3 shows this variation between the two nutrient regimes. In no cases were there detectable (univariate) width or height differences attributable to the presence of pea crabs.

Shape analysis

Using the 13 elliptic Fourier coefficients as shape descriptors, we computed the mean shell shapes for mussels with and without large pea crabs. Mahalanobis' generalized distance D^2 between the multivariate means of these two groups is 2.62; the probability of observing such a large value by chance alone was estimated in a randomization test involving 370 trials to be lower than 0.005. Thus, even though they are probably indistinguishable to a casual human observer, the shell shapes are statistically significantly different between the two groups.

A more conservative test for difference in shell shape between groups with and without pea crabs is a multivariate analysis of covariance that accounts for allometry of shape with mussel size. We performed a mancova on these two classes of shells with length as the covariate and the 13 shape coefficients as the dependent variables. For Wilk's criterion, we observed an F-value of 2.61 for an overall effect of classification with respect to the presence or absence of a large pea crab. This value is significant $P = 0.005$. We therefore conclude that the presence of a pea crab is associated with a change in shell shape. The overall shell length effect is not significant ($F = 0.99$; $P > 0.47$). Apparently, the arithmetic normalization for the size of shell made in the elliptic Fourier analysis removed any statistically detectable allometry from the shape variables.

Figure 4 displays the mussel shapes distributed along the major discriminant axis. This arrangement shows that ungulate mussels indeed separate from spatulate ones

TABLE I

Sample [illegible] ments (in length, width and height) and initial shell length for classes of mussels

	N	Length	Δl	Δw	Δh
[illegible] crab					
with	40	65.3	2.5	.4	.6
without	60	65.6	3.0	.4	.7
****Site*					
good	72	66.0	2.9	.4	.6
poor	28	64.2	2.4	.2	.7
Total	100	65.4	2.8	.4	.7

Units are millimeters. Asterisks indicate the level of significance for the factor.

along the direction that, in a linear sense, best distinguishes mussels that harbor pea crabs from those that do not. This pattern corresponds to the expectation derived from Seed (1968) under the assumption that harboring a pea crab is in fact associated with a reduced growth rate.

DISCUSSION

The nature of the host-crab relationship has been the subject of much speculation in the scientific literature for over forty years. Most evidence to date implies that the pea crab is not the desirable "guardian of the Pinna" as Aristotle claimed (see Thompson, 1910, p. 547b), but is more of a parasite than a commensal. Gill lesions, found in infested *Mytilus edulis* (McDermott, 1962) and *Crassostrea virginica* (Flower and McDermott, 1952; Christensen and McDermott, 1958; Haven, 1958), were attributed to the crab's scraping its chelipeds across the host's soft body parts while feeding. Pearce (1966) mentioned an indentation in the gonadal mass of a variety of host clams and mussels. Fibrous lumps or nodules, up to one centimeter in length, have been found on the mantle lips of bivalves harboring pea crabs (Stauber, 1945; Dix, 1973; Jones, 1977). Kruczynski (1972) noted a reduction in dry meat weight of infested scallops when compared with uninfested scallops of the same size. Bivalve meat reduction was greater than the amount corresponding to the physical mass of the pinnotherid. Similarly lowered meat contents were reported for oysters (*C. virginica*) containing *P. ostreum*, and the California mussel (*M. californianus*) containing *Fabia subquadrata* by Haven (1958) and Anderson (1975), respectively. Notched scallops containing pea crabs grew less over a three-month period than uninfested *Argopecten irradians* of similar size. Kruczynski (1975) proved that *Pinnotheres maculatus* ingests the food of *Mytilus edulis* by observing accumulation of ^{14}C in pinnotherid tissue after the host was fed labeled diatoms (*Nitzchia closterium* and *Thalassiosira pseudonana*). Pregenzer (1979) demonstrated that particles of neutral red dye are cleared from water at a slower rate by mussels that contain pea crabs (*Pinnotheres hickmani*). Silas and Alagarswami (1965) reported that a hermaphroditic species of Indian osyters (*Ostrea cucullata*) normally exhibiting a 50:50 sex ratio becomes signficantly skewed toward maleness when infested with pinnotherids. They suggested that the stress on the host allows only sperm production instead of energetically more expensive egg production.

Some researchers have reported that size of pea crab is related to size of the host [*e.g.*, Wells (1940) on *Fabia subquadrata* in the mussel *Modiolus modiolus*, and Lopez (1982) on *Pinnotheres modiolicola* in the mussel *Modiolus metcalfei*]. We calculated

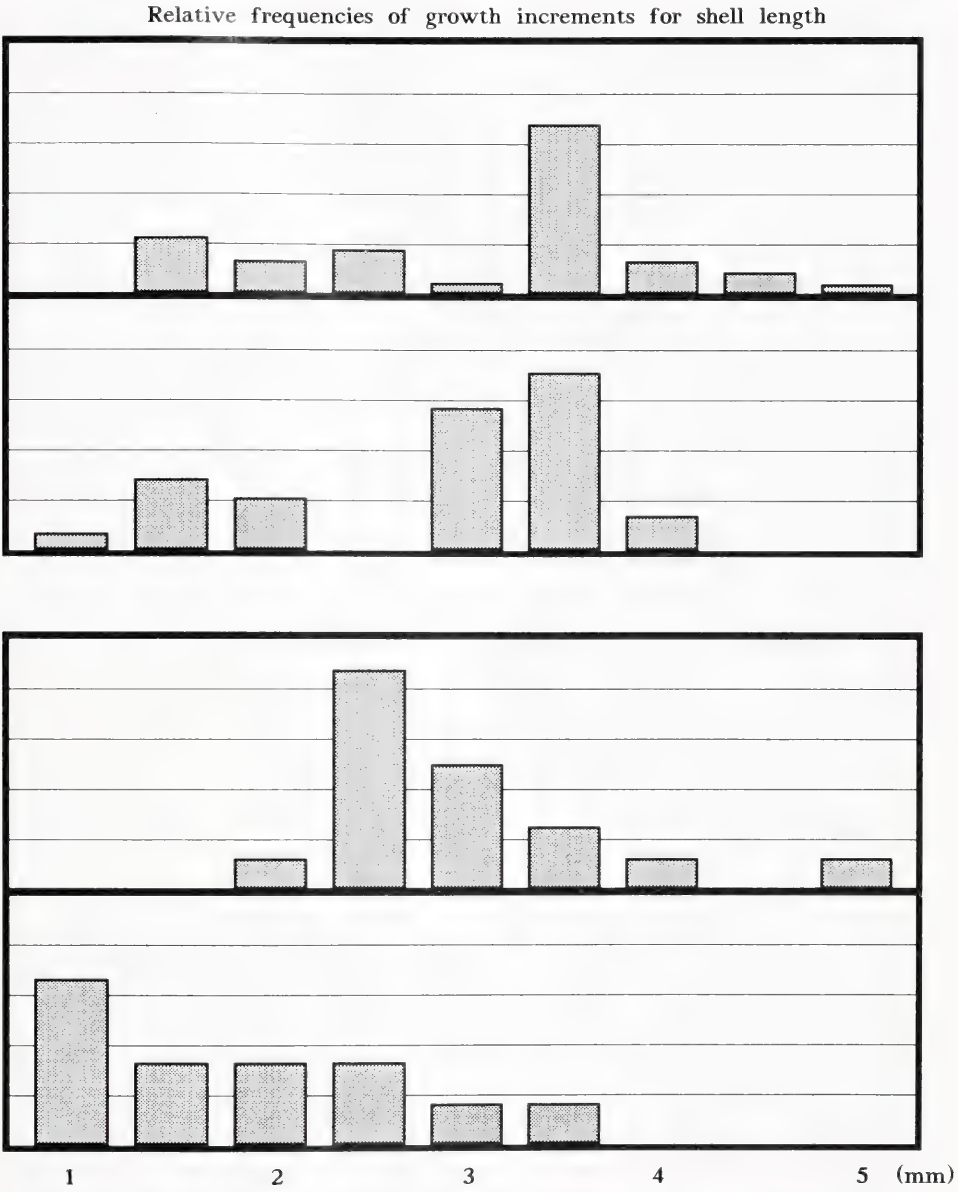

FIGURE 3. Length increment frequencies for mussels in two environments without and with infesting pea crabs. From the top panel, high-nutrient environment without large pea crabs, high-nutrient environment with crabs, low-nutrient environment without crabs, and low-nutrient environment with crabs. Under high-nutrient conditions, there was no significant difference in growth increment between infested and unifested mussels. In low-nutrient conditions, mussels with large pea crabs grew less over a three-month period when measured by change in shell length.

regression lines for several independent populations and found that, if the smallest mussels containing pea crabs are excluded, there is no signficant relationship between size of mussel and size of pea crab. Because small mussels can physically house only small crabs, including them in the regression calculation rotates the best fit line toward

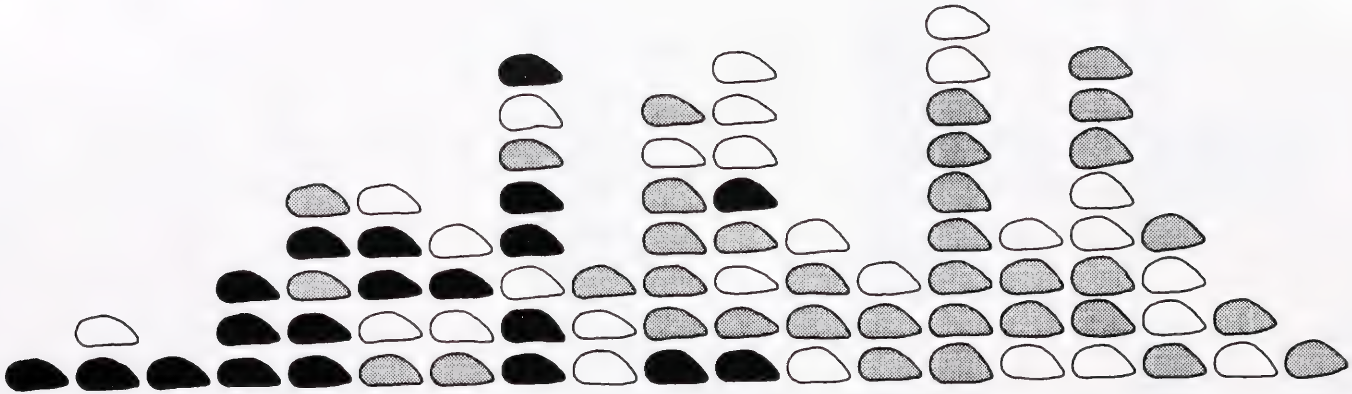

FIGURE 4. The original shell outlines arrayed as a histogram along the linear discriminant axis between shells with and without large pea crabs. Black shells had pea crabs over 6 mm; gray shells had smaller infesting crabs; white shells had no crabs at all. Large crabs are presumably a full year older than small ones and thus have had a chance to influence shell shape in their hosts. Under elliptic Fourier analysis, shells from mussels harboring large pea crabs are significantly different in shape from other mussels. Since these (black) mussels are more elongate, the direction of this shape difference is consistent with the hypothesis that pea crab infestation retards mussel growth.

the origin. Although the regression becomes significant, only 4% of the variation is explained. In the size range of 50 to 90 mm, there is no trend for large *P. maculatus* to be found in larger *M. edulis.*

Our short-term field study indicates that the mere presence/absence of a pea crab may not be the appropriate criterion by which to divide host groups when investigating deleterious effects of pinnotherids. Male and small female pea crabs are not really 'present' over long periods because they can and do move freely from host to host. The fact that a potential host is empty when an experimenter opens it provides no information about the recent past. A large pea crab may have recently died, or a small pea crab may have just moved out. Fortunately, we were able to quantify the frequency of small crab host-switching by suspending infested mussels in a wide mesh cage 2 meters above the substrate. If a pea crab attempts to migrate under these conditions it falls to the ocean bottom and is lost from the population. While a host on the bottom could easily be re-entered, a pea crab cannot readily return to suspended hosts. Work by Pregenzer (1981) corroborates our findings. He artificially implanted *P. hickmani* in a raft population of *M. edulis* and, after 4 months, found 46% of the female pea crabs and 100% of the male pea crabs had vacated their hosts. Unfortunately, he does not report the sizes of female pea crabs either lost or remaining. In our experiment, mussels lost 44% of the total number of crabs present, and 90% of the small (< 6 mm) crabs. Based on this information, we maintain that to isolate long-term effects of pea crabs on host physiology, only adult females should be considered as 'infesting.' The short-term effects of small pea crabs on elastic physiological responses in the host (*e.g.*, oxygen consumption, feeding rate, byssal thread production) remain of interest.

In a three-month experiment, mussels both with and without symbiont pea crabs were grown under two nutrient regimes. Under high-nutrient conditions, there were no statistically significant effects on growth increment due to pea crab infestation for length, width, or height of the mussel host. However, under lower-nutrient conditions, length increment was significantly reduced among mussels harboring large pea crabs compared to their uninfested neighbors. Therefore, in stressful environments, two mussels of the same age would not be the same size if one has been harboring a large pea crab.

External morphology in mussels is known to depend on growth rate. A slowly growing mussel records its growth history in the elongate and recurved or "ungulate" shape of its shell. A fast-growing mussel records its history in a triangular or "spatulate" shell shape. Thus shell form represents an integration of physiological status over several years. To examine the long-term effects of harboring a pea crab, we numerically characterized shell shapes for individuals from Gay Head, Massachusetts, that had been infested with at least year-old pea crabs and for individuals that were free of large pea crabs. The bed was an extensive and robust population from which thousands of individuals had been harvested over a two-year period (F. Valois, Marine Biological Laboratory, pers. comm.). Presumably then this was an environment favorable for mussels. In our analysis we corrected for allometry of shape due to size and therefore captured shape itself. By doing so, we were able to differentiate between slightly ungulate mussel shapes characteristic of the presence of large pea crabs, and more spatulate mussel shapes characteristic of uninfested mussels. Thus over the long-term, pea crabs appear to retard host growth rates under conditions that may be only periodically stressing, or even optimal.

ACKNOWLEDGMENTS

The mussels were collected by the Marine Biological Laboratory, Woods Hole, Massachusetts. We thank F. J. Rohlf at Stony Brook for his advice on many problems and for allowing us access to image analysis equipment and software developed under National Science Foundation Grant DEB 8202269. We also thank L. B. Slobodkin for his advice and his encouragement of this project. R. Nuzzi and M. Waters of the Suffolk County Department of Health very kindly provided us with a large data set pertaining to water quality off Long Island. B. Glass, R. K. Koehn, G. Hechtel, K. [illegible] M. Monahan, and K. Dunn read the manuscript and recommended several improvements. We thank L. Harder for discussions on matters statistical. This is contribution 571 from the Graduate Studies in Ecology and Evolution, State University of New York at Stony Brook.

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PHOTOADAPTATIONS OF THE CARIBBEAN COLONIAL ASCIDIAN-CYANOPHYTE SYMBIOSIS *TRIDIDEMNUM SOLIDUM*

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ABSTRACT

Photosynthetic organisms show a number of photoadaptations which enable them to acclimate to the light regime in which they grow. In the case of invertebrate-algal symbioses, the responses can occur in both the algae and the invertebrate. Colonies of the colonial ascidian-cyanophyte symbiosis *Trididemnum solidum* at Galeta, Panama, show morphological variation relative to the light regime in which they live. Colonies growing in full sunlight are white, thicker, more heavily calcified, and distribute their cyanophytes more uniformly throughout the depth of the colony than do colonies in lower light regimes. Shaded colonies are purple, thinner, have fewer spicules at the colony surface, concentrate their algae near the surface, and have a greater amount of phycoerythrin relative to phycocyanin in their symbiotic algae. The purple coloration of low light colonies appears to be due primarily to the phycobilin pigment of the algae. Experimental shading of colonies in the lowest light areas in which they are found at Galeta, resulted in significantly higher mortality than colonies beneath clear control roofs. The ascidian host appears to be physiologically dependent upon its symbionts and capable of changing its morphology in response to ambient light levels.

INTRODUCTION

Symbioses with unicellular algae occur in a wide range of marine invertebrates from single cell protozoa (Karakashian, 1963) to the giant clams, *Tridacna* (Taylor, 1973). Most research on such symbioses has examined eucaryotic symbionts (zooxanthellae, zoochlorellae) in the tisues of anthozoan coelenterates and other invertebrates (reviewed by Trench, 1980). However, the occurrence of procaryotic symbionts has been noted in sponges (Sara, 1971; Vacelet, 1971; Wilkinson, 1982) and several species of ascidians (Lewin, 1975).

Although the existence of colonial ascidians with symbiotic algae has been known for over seventy years (Herdman, 1907), the fact that the symbionts are procaryotes was not realized until 1975 (Newcomb and Pugh, 1975). The algae had previously been mistakenly identified as zooxanthellae (Hastings, 1931; Smith, 1935) or zoochlorellae (Smith, 1935; Tokioka, 1967). After an extensive analysis of the algae associated with didemnid ascidians of the Pacific, Newcomb and Pugh (1975) concluded that "some or all of the zoochlorellae reported in ascidians by past workers may have been blue-green algae." This belief is corroborated by Lafargue and Duclaux (1979).

Lewin (1975) described a unique group of symbiotic algae found only in association with didemnid ascidians. These algae possess traits which do not conform to the current taxonomy of unicellular algae. They contain chlorophyll b, lack phycobilin pigments,

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Received 8 August 1985; accepted 25 November 1985.

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and occasionally have stacked thylakoids (Thorne *et al.*, 1977; Giddings *et al.*, 1980). This suggests that they are eucaryotes, perhaps chlorophytes. However, they have a typical cyanophyte structure without a nuclear membrane or membranous organelles, and with a cell wall that has four layers (Whatley, 1977) and the peptidoglycan complex that characterizes procaryotes (Moriarty, 1979). Lewin (1977) has established the Prochlorophyta as a new division, with *Prochloron* as the type genus.

Of the 20 species of ascidians obligately associated with symbiotic algae (Kott, 1982), 17 possess algae of the newly described genus *Prochloron* (Lewin, 1975). The other three species (*Trididemnum solidum, T. Cyanophorum,* and *Didemnum viride*) contain symbionts of the genus *Synechocystis,* a true cyanophyte with phycobilin photosynthetic pigments (Lafargue and Duclaux, 1979; Kott, 1980). These three species range in color from grey to purple depending upon the light regime in which they grow (Olson, 1980).

The ecology and physiology of *Trididemnum solidum,* a species found only in the Caribbean, was investigated in Curacao and reported by Bak *et al.* (1981), van Duyl *et al.* (1981), and Sybesma *et al.* (1981). In this paper I present data on *T. solidum,* from Galeta, Panama, documenting the changes in photosynthetic pigments and colony morphology between high and low light environments, and the results of an experiment which demonstrates the obligate nature of this symbiosis. For simplicity, I have chosen to use the term "algae" to refer to the *Synechocystis* cells, even though they are actually cyanophytes.

MATERIALS AND METHODS

Field research was conducted on the fore reef at Galeta, Panama, in front of the Galeta Marine Laboratory. The Galeta reef is adjacent to mangroves and has a heavy sediment load (Cubit and Williams, 1983). *T. solidum* occurs from the low intertidal zone to approximately 8 m depth around Galeta.

Colony morphology

In this paper the term colony is used to refer to a single specimen of *T. solidum* composed of physiologically attached zooids. Colonies were collected from a full sunlight habitat (less than 1 m depth, unshaded; these will be referred to as "light colonies") and a shade habitat (2 m depth, shaded by overhanging ledge; referred to as "shade colonies"). A 0.1 m^2 quadrat was placed over a large aggregation of colonies, then starting at one side, all colonies were collected until 50 colonies were obtained. Colonies were gently peeled off the coral rubble on which they grew. A few colonies had fragments of rubble or algae attached to their tunic, but these were easily cleaned off. To measure upper surface area, colonies were laid flat on paper and outlined, then the outline was measured with a planimeter. Colony volume was measured by displacement of seawater in a graduated cylinder. Colonies were dried at 60°C for 24 hours and weighed. To obtain ash-free dry weights, colonies were combusted in a muffle furnace at 500°C for 12 hours and weighed.

Histology

Colonies were fixed in 5 percent Bouins solution and kept for 3 months, during which time they decalcified. Specimens were embedded in paraffin and 5 μm sections were cut. Sections were stained with hematoxylin and fast green. Algal distribution "transects" were performed by examining slides under a compound microscope with an ocular grid. Three randomly selected transects were performed on one section each from four light colonies and four shade colonies.

No attempt was made to quantify algal cell densities in terms of surface area of colony or biomass. This is because the algae are firmly embedded in the tunicin-mucopolysaccharide matrix of the tunic. Maceration and homogenization failed to produce any reliable separation of algal and animal tissue.

Photosynthetic pigments

Specimens for pigment extraction were frozen at −20°C and transported to Cambridge, Massachusetts, in dry ice kept in total darkness. Colonies were sampled by cutting a square core of 1 cm × 1 cm from the top of the colony to the bottom using a razor blade. Extractions were performed for chlorophylls and phycobilins using separate samples for each. The squares were ground to a powder in a mortar and pestle with dry ice. Phycobilin pigments were extracted in 10 ml of 0.01 M $NaHPO_4$, 0.15 M NaCl, pH = 7.0. Chlorophyll *a* and carotenoids were extracted in 25 ml of 90% acetone. The samples were centrifuged at 14,000 rpm for 20 minutes, then analyzed on a Cary 219 scanning spectrophotometer. Absorbance maxima for phycoerythrin and phycocyanin were read at 545 and 615 nm, respectively. Phycobilin pigment concentrations were calculated with the equations of Bennett and Bogorad (1973). Chlorophyll *a* concentration and total carotenoids were calculated using the equations of Jeffrey and Humphrey (1975), and Strickland and Parsons (1965), respectively.

Shading experiment

To determine whether *T. solidum* is physiologically dependent upon its symbiotic algae, the supply of sunlight to colonies was altered *in situ* using ¼" thick plexiglas roofs. Six pairs of 20 cm by 20 cm roofs, consisting of one clear roof (control) and one black roof (shade) were mounted approximately 2 cm above the substratum. The locations chosen were areas of near 100 percent space cover by *T. solidum* and were at the very lowest light intensities that *T. solidum* is found at Galeta (approx. 5 m depth).

The roofs were fastened to the substratum with one nail at each corner attached with plastic cable ties. The area beneath each roof was monitored at the beginning, then after four and six days by removing the roof, and overlaying a piece of clear plexiglas with 100 random dots. Each dot was scored for the presence or absence of *T. solidum* beneath it. The experiment was frequently checked for the presence of organisms seeking refuge beneath the dark roofs, but none was observed.

RESULTS

Colony morphology

Colonies of *T. solidum* on the fore reef at Galeta exhibit a continuum of colors from white to gray to purple (Figs. 1, 2). The coloration of colonies closely corresponds to the habitat in which the colonies live. Colonies in full sunlight, shallow sites were bright white and bulbous, packed with calcareous spicules (Fig. 3), to the top of the colony and slightly larger (Fig. 4) than shaded colonies. Colonies in shaded habitats were dark purple, with few spicules in the top 1 mm of the tunic. The thickness of light colonies ($\bar{x} = 3.28$ mm, S.E. = 0.088, n = 50) was significantly greater than shade colonies ($\bar{x} = 2.91$ mm, S.E. = 0.083, n = 50) (*t*-test, $P < 0.005$).

The difference in calcification between shade and light colonies is shown by the difference in ash-free dry weights. Light and shade colonies had mean percent ash-

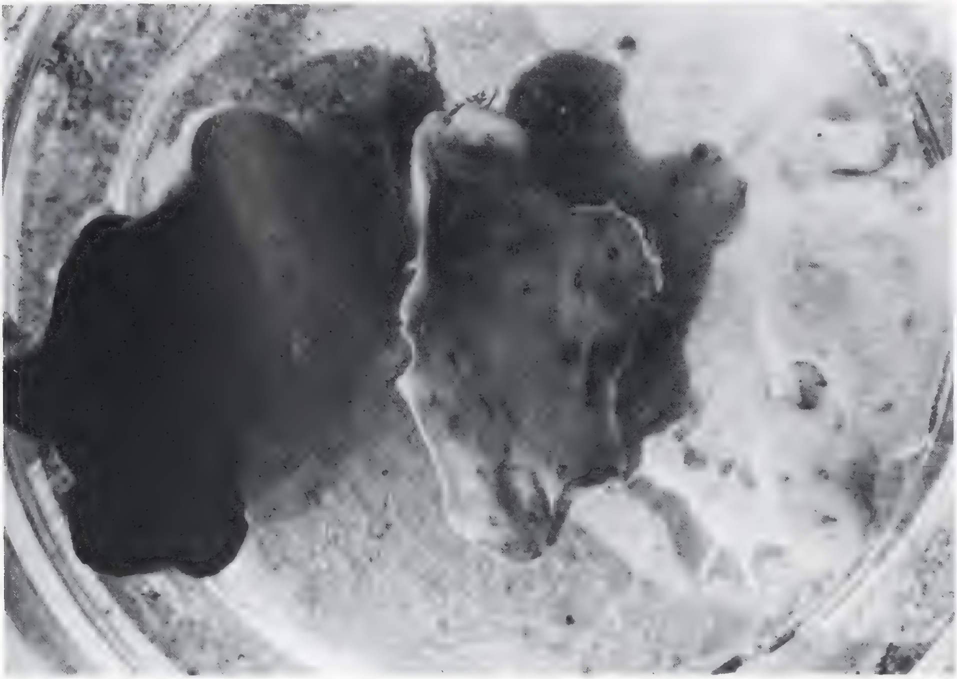

FIGURE 1. Top view of *Trididemnum solidum* colonies collected from (left to right) low, medium, and high light intensity habitats. Colonies are approximately 10 cm in length.

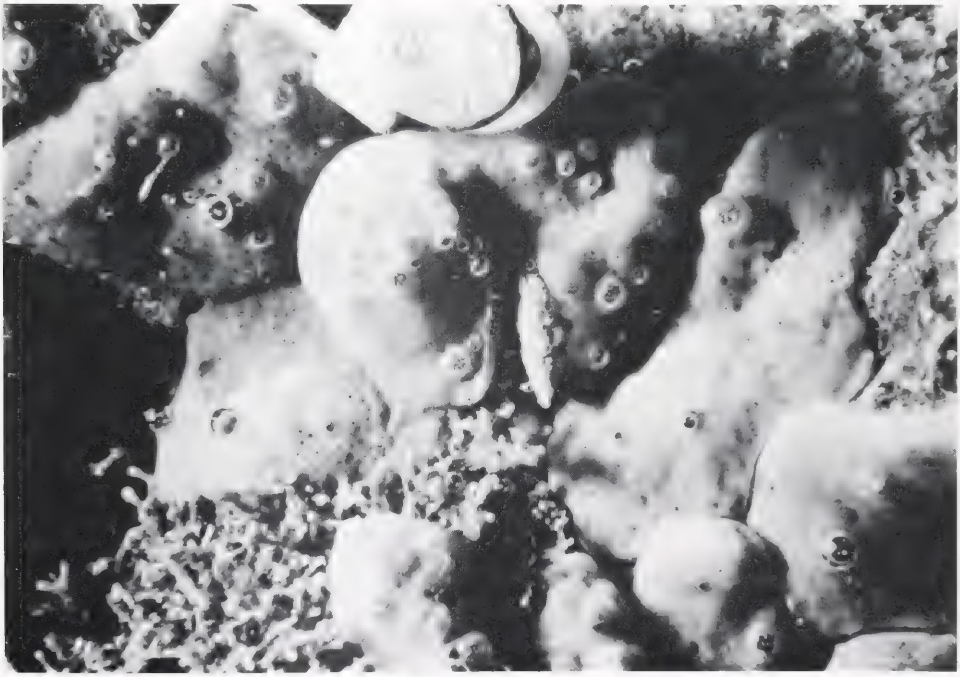

FIGURE 2. Colonies in a full sunlight habitat (<1 m depth) at midday. Note oxygen bubbles trapped within tunic. Small white specs on bubbles are calcareous spicules.

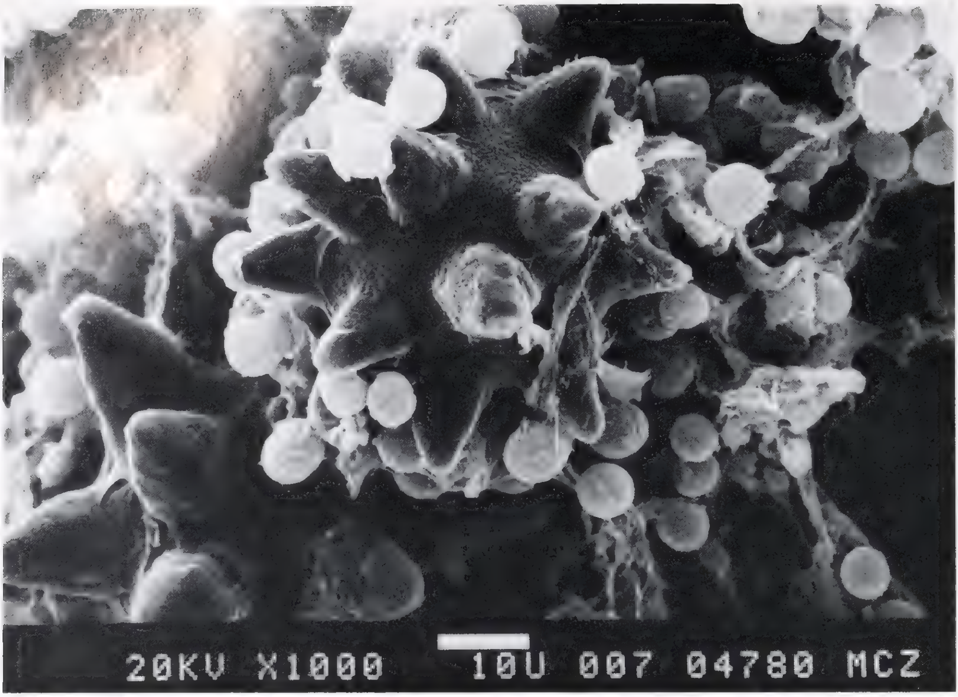

FIGURE 3. Scanning electron micrograph of calcareous, stellate spicules and *Synechocystis* algae (small spheres) in the tunic of *T. solidum*. Top portion of photo is top of colony. (Photo by E. Seling.)

free dry weights (ash-free dry weight/total dry weight) of 13.28 (S.D. = 1.42; n = 9) and 15.46 (S.D. = 1.52; n = 9), respectively. Thus light colonies have a significantly greater inorganic component (*t*-test, $P < 0.01$). This difference is also reflected in the ratio of volume to dry weight (Table I) which shows that light colonies have a greater density than shade colonies. It appears that much of the difference could be accounted for by the top layer of the colonies. Very few, if any, spicules can be seen in the top 2–3 mm of shade colonies, whereas light colonies are generally packed to the top with spicules. Cross sections revealed a zone at the top of light colonies with very little algae (Fig. 5).

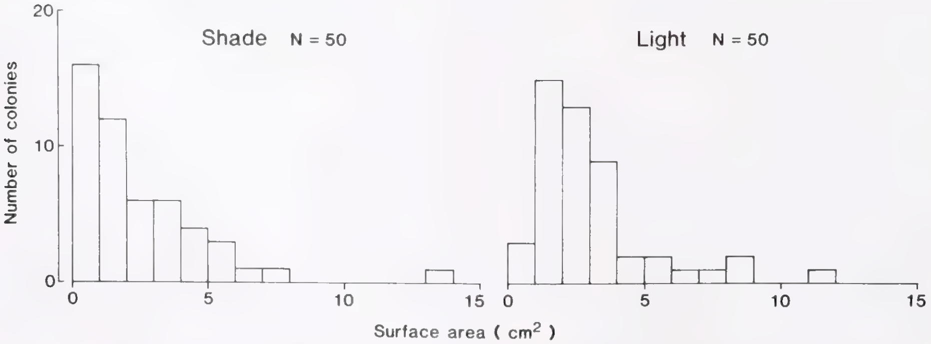

FIGURE 4. Size distribution, by surface area, of colonies from shade and full sunlight locations.

TABLE I

Regression analysis of colony morphology parameters. Comparison of slopes and elevations performed with t-*test (Zar, 1974)*

	Light				Shade				Level of significance	
Regression (y *vs.* x)	n	Slope	Y-intercept	r^2	n	Slope	Y-intercept	r^2	Slopes	Y-int.
y x										
Volume *vs.* dry weight	50	1.80	0.014	0.91	50	1.82	0.132	0.87	N.S.	$P < 0.005$
Volume *vs.* upper surface area	50	0.187	0.029	0.80	50	0.134	0.110	0.86	$P < 0.0005$	N.S.
Upper surface *vs.* dry weight	50	8.50	0.314	0.89	50	13.15	0.233	0.96	$P < 0.01$	$P < 0.01$

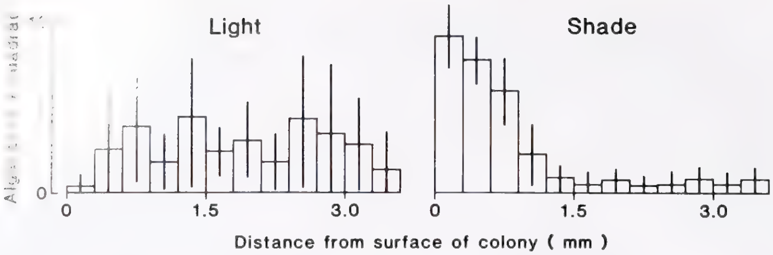

FIGURE 5. Distribution of algae through depth of colonies from sections of shade versus full sunlight colonies. n = 3 transects from 4 colonies each. Error bars are standard errors. Algal abundance is expressed as a relative measure (# cells/quadrat).

Photoadaptation

The difference in coloration of colonies (Fig. 1) is primarily due to their levels of phycobilin pigments. The algae of dissected light colonies are light brown in color, those of shade colonies are dark purple, almost black. There was twice as much phycoerythrin per unit of surface area in shade colonies than in sun colonies (Table II). Aqueous extracts of shade colonies were red in color *versus* light pink for sunlight colonies. Shade colonies also had a greater mean concentration of phycocyanin than light colonies.

In addition to the differences in the amount of phycobilin pigment, extracts from the sun colonies had much greater absorbance in the ultraviolet-B region, with a peak absorbance around 330 nm. The difference in absorbance down to 400 nm can be seen in Figure 6.

Shading experiment

During the six days of the shading experiment, colonies beneath the dark roofs contracted and most began to deteriorate. Of the approximately 100 colonies beneath

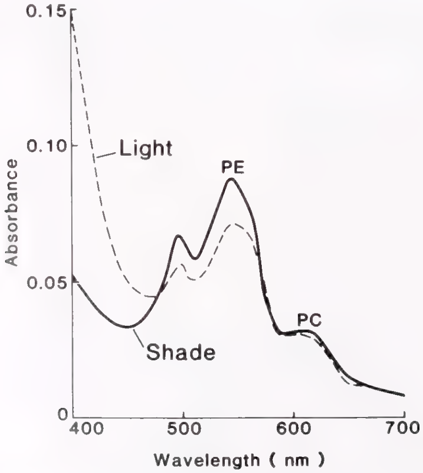

FIGURE 6. Absorbance spectra of water soluble pigments from representative light and shade colonies. PC—phycocyanin, PE—phycoerythrin.

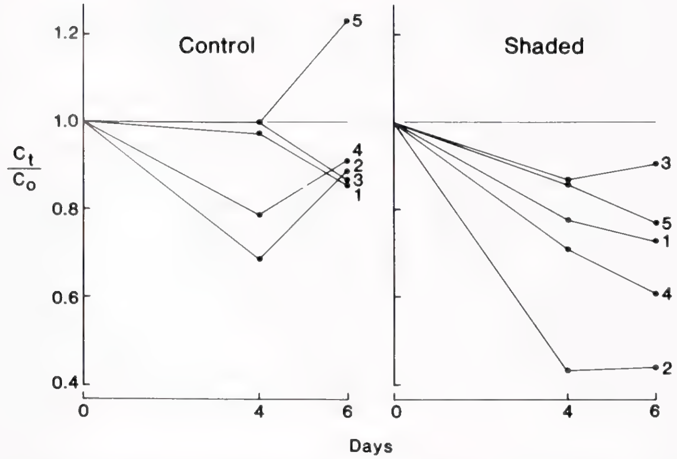

FIGURE 7. Results of shading experiment. Data are % cover of *Tridideminum solidum* at time t (C_t) divided by initial % cover (C_0). Treatments were paired according to numbers.

the dark roofs, twelve entire colonies died. No sign of tissue death was observed in the colonies beneath the clear roofs. The mean relative change in percent cover of colonies beneath the shade treatments (−31.1%, S.D. = 0.17) was significantly greater than the relative change in percent cover of the colonies beneath the controls (−4.9%, S.D. = 0.16) ($P < 0.05$, Wilcoxon paired sampled test) (Fig. 7).

As colonies began to die beneath the shades, they turned green for a day or two, then white. The change to green is most likely due to the breakdown of the phycobilin pigments, removing the purple coloration. The sky was very cloudy during the experiment, which might account for the decline in percent cover of the control colonies. The breakdown of phycobilin pigments in stressed algae is documented for laboratory cultures of blue-green algae such as *Anabaena* (Ownby *et al.*, 1979) and *Synechococcus* (Yamanaka and Glazer, 1980). The white material remaining after about 3 days was primarily spicules.

DISCUSSION

Photoadaptation

Photosynthetic organisms acclimate to the light regime in which they grow primarily through changes in: (1) the production of photosynthetic pigments, (2) the structure of chloroplasts, and (3) the photosynthetic response (Boardman, 1977). Studies of photoadaptation in invertebrate-algal symbioses have focused on eucaryotic cells, particularly the dinoflagellates found in symbiosis with corals (Titlyanov *et al.*, 1980; Zvalinskii *et al.*, 1980; Falkowski and Dubinsky, 1981; Dustan, 1982; Chalker *et al.*, 1984; Kinzie *et al.*, 1984; Porter *et al.*, 1984).

Trididemnum solidum colonies from full sunlight *versus* shaded habitats at Galeta showed the following five differences:

(1) *Distribution of algae within colony*

Algae in the tunic of shade colonies occurred primarily in the top 1 mm of the colony (Fig. 5). This packing of algae near the surface appears to be analogous to the arrangements of chloroplasts in cells of shaded-adapted terrestrial plants (Boardman, 1977). Algae [illegible] at the top of the colony would be exposed to the maximum level of light available. The mechanism through which the algae arrive at this location is unknown.

(2) *Ratio of organic to inorganic matter*

Shade colonies have a greater proportion of organic matter, as shown by the ratio of ash-free dry weight to dry weight. Light colonies have more spicules and are more dense (Table I). This same response has been reported for other colonial ascidian-algal associations. Kott (1980) reported that colonies of *Lissoclinum voeltzkowi* on the Great Barrier Reef produce a more dense layer of spicules at the surface of the colony when growing in full sunlight than when growing in the shade. *Didemnum viride* (another ascidian-*Synechocystis* association) also shows less dense spicules in shaded habitats (pers. obs.). Newly settled larvae of *Didemnum molle* produce few spicules if shaded, but produce a dense layer of spicules if grown in full sunlight (Olson, 1984).

There are at least three reasonable hypotheses as to why spicule production is directly related to sunlight intensity. Spicule production might be (1) an ecological response to predators because high light intensity habitats are usually exposed to a wider variety of predators, or (2) it might be used to regulate the amount of sunlight entering the colony, or (3) it could be a physiological consequence of photosynthesis by symbiotic algae. These hypotheses are not mutually exclusive.

Field observations suggest that predation might be an important factor. *T. solidum* colonies grow in fully exposed habitats at Galeta, yet no fish were ever observed to prey on them. The only predators ever observed were a flatworm and occasionally, the sea urchin *Diadema antillarum*.

Spicules in the surface layer must function to some extent to reduce the amount of sunlight entering the colony. The distribution of algae through the depth of the colony (Fig. 5) suggests that light levels in full sunlight habitats might be super-saturating. The presence of spicules and U.V. absorbing compounds might protect the algae. In shaded habitats, having spicules in the top of the colony would reduce light penetration, shading algae below.

The third hypothesis was postulated for corals by Goreau (1959). Light enhanced calcification has been documented extensively for corals (see Chalker, 1983, for review). Increased spicule production in *T. solidum* growing in full sunlight might simply be a physiological consequence of the ambient light regime. However, among the ascidian-algal symbioses there are three species, all in the genus *Diplosoma,* which lack spicules despite the presence of symbiotic algae (Kott, 1980). There are also a wide range of didemnid ascidians which lack symbiotic algae, yet possess calcareous spicules (Eldredge, 1967). This casts doubt on the physiological consequence hypothesis.

(3) *Photosynthetic pigments*

The most significant, and obvious, difference in photosynthetic pigments between light and shade colonies was the phycoerythrin content (Table II). Phycoerythrin is an accessory photosynthetic pigment which has an absorbance maximum in the green light region around 560 nm (Bennett and Bogorad, 1973). Increased production of this pigment in the symbiotic algae of shade colonies follows the classic chromatic adaptation of blue-green algae described by Fujito and Hattori (1960, 1962). Cyanophytes grown in green light produce greater amounts of phycoerythrin. Those grown in red light produce more of the red light absorbing pigment phycocyanin. This results in different coloration of the algae growing in different light spectra, as appears to be the case with the *Synechocystis* of *T. solidum*.

TABLE II

Mean concentrations of photosynthetic pigments per unit area of T. solidum *colonies from light and shade habitats*

	Light		Shade		Level of significance*
Chlorophyll a ($\mu g/cm^2$)	98.73	(20.89)	110.65	(12.85)	N.S.
Total Carotenoid ($\mu g/cm^2$)	26.08	(4.71)	23.13	(2.97)	N.S.
Carotenoid/Chlorophyll *a*	0.27	(0.03)	0.21	(0.006)	$P < 0.001$
Phycocyanin ($\mu g/cm^2$)	69.0	(14.48)	105.0	(34.25)	$P < 0.05$
Phycoerythrin ($\mu g/cm^2$)	68.3	(16.56)	155.0	(39.9)	$P < 0.001$
Phycocyanin/Phycoerythrin	1.02	(0.12)	0.67	(0.14)	$P < 0.005$

Values in parentheses are standard deviations. n = 6 colonies for all values. **t*-test.

Kinzie *et al.* (1984) have emphasized an important distinction in photoadaptation; whether the response is to light quality (spectral composition) or light intensity. Since dark purple *T. solidum* colonies can be found in shaded sites at shallow depths (1–2 m) and colonies in unshaded habitats at deeper depth (4–5 m) have the light appearance, it would appear that the responses are largely to decreased light intensity. This aspect awaits further investigation.

The amount of chlorophyll *a* per cm^2 was slightly greater in shade colonies (Table II) although the difference was not significant due to the large variation. Shade-adapted algae generally have a greater amount of chlorophyll *a* per cell (Porter *et al.*, 1984). However, Sybesma *et al.* (1981) also found no significant difference in chlorophyll *a* content of colonies growing at different light levels. The values I obtained for chlorophyll *a* were approximately four times greater than those found by Sybesma *et al.* (1981) for *T. solidum* in Curacao, and are approximately ten times greater than values reported by Porter *et al.* (1984) for corals.

Although the total amount of carotenoids did not differ between light and shade colonies (Table II), there was a significantly higher ratio of total carotenoids/chlorophyll *a* for light colonies. This is consistent with what is known for terrestrial plants, in which it is suggested that carotenoids might serve to protect chlorophyll *a* from photodegradation (Robertson *et al.*, 1966).

(4) *Colony size and thickness*

Colonies in full sunlight were slightly larger (Fig. 2) and thicker. This change in colony morphology resembles the variation with depth that is seen with reef-building corals (Dustan, 1975; Redalje, 1976; Foster, 1979) in which colonies from greater depth, growing in lower light, assume a thinner, more plating morphology.

(5) *U.V. absorbing compounds*

Though ultraviolet radiation penetrates little more than the first 10 m of the ocean (Jerlov, 1968), it is known to inhibit photosynthesis (Smith *et al.*, 1980) and can be lethal to invertebrates in shallow water (Jokiel, 1980). *T. solidum* at Galeta is abundant in exposed areas all the way up to the low tide level. Light colonies experience almost the full level of surface U.V. irradiation and thus must somehow be protected from this. The aqueous extracts of colonies (Fig. 6) showed the presence of U.V. absorbing compounds in much greater quantity in light colonies than shade. This peak had a maximum at approximately 330 nm, the upper end of the U.V.-B region (Smith and Baker, 1979). The compound or compounds responsible for the absorption probably function in a manner similar to the "S-320" substances isolated from corals (Shibata, 1969).

The importance of sunlight

The shading experiment (Fig. 7) indicates that the ascidian host is dependent upon the well-being of its symbiotic algae. Reduction of light, beyond a certain level, is lethal to the colony. Whether the death of the colony is caused by starvation from insufficient production by the algae, or is due to toxicity of the dying algae, is unclear. Regardless, both symbionts are unable to survive without sunlight. Thus the distribution of *T. solidum* should be limited to habitats in which sunlight is in excess of the minimum level needed for the symbiotic algae. On the other hand, there does not appear to be any maximum light level at which colonies can survive since they occur in the low intertidal, unshaded, on the Galeta reef flat.

Considerable data already exist on the ecology and physiology of *Trididemnum solidum* in Curacao (Bak *et al.,* 1981; Duyl *et al.,* 1981; Sybesma *et al.,* 1981). It is important to note the substantial differences (particularly colony size) between that population and the population of Galeta, Panama. Colonies from Curacao are as large as 50 cm in diameter. Almost all of the colonies reported by Bak *et al.* (1981) were at least 15 cm diameter, or about 175 cm^2 surface area (assuming a circular shape). In contrast, Panama colonies were rarely more than 10 cm in length, and the surface area of the largest colony collected for the colony morphology analysis was 14 cm^2 (Fig. 2).

Colonies of *T. solidum* at Galeta divide very rapidly (taking only one day at times) producing a "jigsaw puzzle" of thousands of colonies abutting each other, and covering areas of several square m. Whereas a single Curacao colony might cover 0.25 m^2, at Galeta I was able to collect all 50 colonies for the colony morphology measurements in less than 0.1 m^2 area.

Although both populations have been identified by R. H. Millar as *T. solidum* (Cubit and Williams, 1983; Duyl *et al.,* 1981), there appear to be differences in their reproductive patterns. In Curacao, colony fusion occurs almost as frequently as colony division, so there is little asexual propagation (Bak *et al.,* 1981). Although I watched closely for it, I never observed colony fusion in *T. solidum* at Galeta. More than 50% of all colonies produced larvae throughout the year in Curacao (Duyl *et al.,* 1981). At Galeta, Millar (1974) found that for half of the year (January–June) no colonies produced larvae.

The difference in colony size between the Galeta population and the populations in Curacao (Bak *et al.,* 1981), Puerto Rico, and St. Croix (pers. obs.) poses an interesting question. Is this difference due to a divergence in life history patterns, with colonies in the Galeta population maximizing the number of distinct colonies within a genetic individual? Or is there a physiological reason for colonies maintaining a small size, such as hydrodynamics (Vogel, 1981) or food availability (Sebens, 1979)? The first response of the shaded colonies, in the shading experiment, was to shrink and divide. Stoner (1985) found that colonies of the ascidian-*Prochloron* symbiosis *Diplosoma similis* divided at smaller sizes when grown at low light levels. Water clarity at Galeta for much of the year is considerably lower than for the islands of Curacao, Puerto Rico, and St. Croix. The Galeta reef, being adjacent to mangroves, has a heavy sediment load and water clarity is extremely low for most of the dry season [December to April (Millar, 1974)]. Reduced light penetration would reduce the productivity of the symbiotic algae, possibly reducing the supply of energy available to the ascidian host. Perhaps the more stressful physical conditions at Galeta have selected for a morph of *T. solidum* with colony division at a smaller size.

ACKNOWLEDGMENTS

This research was supported by a Smithsonian Tropical Research Institute short-term fellowship and the Anderson Fund (Harvard University). I thank J. Cubit, E. Gantt, M. Hay, J. Kilar, T. Newberry, K. Sebens, E. Seling, B. Thorne, C. Wilkinson, and R. Woollacott for assistance and helpful discussion. Access to facilities at the Galeta Marine Laboratory was made possible by the Smithsonian Institution Environmental Sciences Program. The Australian Institute of Marine Science provided facilities for the analysis of the data, and M. Thyssen helped with the figures. The proding and encouragement of M. Patterson and my wife Molly helped bring this paper to fruition. The comments of one anonymous reviewer were greatly appreciated.

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EFFECTS OF STRONTIUM ON THE EMBRYONIC DEVELOPMENT OF *APLYSIA CALIFORNICA*

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ABSTRACT

Strontium is required in defined media for the development of normal shells and statoliths in the opisthobranch gastropod, *Aplysia californica* Cooper, 1863. Embryos grown in artificial seawater without strontium chloride become larvae with normally appearing soft tissue but with deformed shells and statocysts that lack the statolith granule. Shell and statolith formation is sensitive to concentration differences as small as one part per million strontium in the artificial seawater. Pulse experiments indicate a critical period of exposure for the requirement beginning at Day 4, (72 h after oviposition) and lasting no longer than 24 h. Animals exposed to artificial seawater containing 8 parts per million strontium only during this critical period develop normally. Conversely, animals immersed in artificial seawater lacking strontium during this same period, but exposed to the element during the rest of the 10-day embryonic phase, exhibit anomalous shells and statocysts. The effects are only marginally reversible if strontium is presented after Day 5. Strontium is also essential for the normal development of locomotory behavior. Animals that have been deprived of strontium swim erratically, spinning in tight circles. The pathogenesis of this behavioral disorder is explained by the lack of strontium during embryonic development which causes the major structural anomalies at the larval stage. The absence of the statolith results in a defective organ of balance which in turn causes the severe behavioral symptoms. A similar strontium effect in other molluscs, including *Hermissenda crassicornis* (Eschscholtz, 1831), is also documented.

INTRODUCTION

The presence of strontium in the molluscan shell may serve as a record of past and present-day environments (Nelson, 1962; Rucker and Valentine, 1966; Lee and Wilson, 1969), as an indicator of the physiological state of the animal (Dodd, 1965), and as a clue to evolutionary relationships (Lowenstam, 1964). Before the significance of this element's presence can be fully interpreted, however, it will be necessary to disentangle the effects of the various factors that control the strontium content of skeletal carbonates, *i.e.,* water chemistry, skeletal mineralogy, physiology, and environment (Dodd, 1967). The amount of strontium in bivalve and gastropod shells is among the lowest of invertebrate mineralized tissues, suggesting that these animals have an exceptional ability to discriminate against this element (Dodd, 1967). A fundamental question then arises: is strontium required by any molluscan form, and if so, what is its specific biological role?

In the course of developing a simple artificial seawater capable of supporting growth and development of the opisthobranch mollusc, *Aplysia californica* Cooper, 1863, we

Received 2 April 1985; accepted 17 October 1985.

found that the presence of strontium is essential during embryonic development for normal shell and statolith formation. The statocysts, sense organs of spatial orientation, are the earliest neural structures to develop (Kandel, 1979), appearing when the embryo reaches the 300-cell stage. At this time the shell-gland has invaginated to form a narrow pit and soon after everts to form the shell cap which secretes the shell (Saunders and Poole, 1910). Embryonic development in *Aplysia* spp. occurs within the protection of an egg mass, deposited after fertilization by the hermaphroditic adult. The mass is composed of a long, thin gelatinous cordon, which surrounds transparent capsules, each containing from 18 to 20 fertilized eggs. Development is complete 8–12 days after fertilization at 20°C when the free-swimming, planktotrophic veligers emerge from the strand (Kandel, 1979). We report here the effects of varying the strontium concentration in artificial seawater on the embryonic development of *A. californica,* and describe the results of experiments designed to ascertain the critical period of exposure. Results of preliminary experiments with the nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) are also presented.

MATERIALS AND METHODS

The basal medium used reflects the concentrations and relative proportions of the major constituents of natural seawater (Brewer, 1975), consisting of, in grams/liter, 23.88 NaCl; 10.68, $MgCl_2 \cdot 6H_2O$; 4.01, Na_2SO_4; 1.51, $CaCl_2 \cdot 2H_2O$; 0.725, KCl; 0.196, $NaHCO_3$; 0.086, NaBr (pH 8.10 and salinity 34‰). This artificial seawater was prepared from concentrated stock solutions of each reagent grade constituent dissolved in deionized water. The magnesium and calcium stock solutions were standardized by Mohr titration (Skoog and West, 1965). Strontium contamination of reagents, as listed by lot analysis (Fisher), ranged from 0.0 to 0.1%. Subsequent flame atomic absorption measurements failed to detect this element in the basal medium. Strontium is a conservative element of natural seawater and is present at a concentration of $9.10 \cdot 10^{-5}$ *M* at a salinity of 35‰ (Brewer, 1975). We varied the concentration of strontium from 0.0 to $9.07 \cdot 10^{-5}$ *M* (approximately 0–8 ppm in 1 ppm increments) through additions of strontium chloride.

In one experiment we used the basal medium plus H_3BO_3 (25 ppm); $NaH_2PO_4 \cdot H_2O$ (1 ppm, PO_4^{-3}); NaF (1 ppm, F^-); $MnSO_4 \cdot H_2O$ (1 ppm, Mn^{+2}); $Na_2MoO_4 \cdot 2H_2O$ (0.7 ppm, MoO_4^{-2}); LiCl (0.2 ppm, Li^+); RbCl (0.1 ppm, Rb^+); $Al_2(SO_4)_3 \cdot 18H_2O$ (0.04 ppm, Al^{+3}); and KI (0.07 ppm, I^-). This is a modification of a medium described by Segedi and Kelley (1964), an early version of the commercial product Instant Ocean® (Aquarium Systems, Mentor, Ohio) (Bidwell and Spotte, 1985).

Egg masses were obtained from breeding pairs of adult *A. californica,* maintained in a flow-through natural seawater system at Marine Biological Laboratory, Woods Hole, Massachusetts, with a pH range of 7.8–8.2 and a salinity of 31 ± 1‰. Each egg mass was washed in basal medium, cut into strands 1 cm in length, and distributed into sterile, plastic petri dishes, 12–14 strands per dish, equivalent to 40,000–50,000 eggs. Each dish was filled with approximately 50 ml of the experimental medium (enough to cover the strands completely) and incubated at 21°C under continuous illumination. Replicates were prepared for all test solutions and the natural seawater control. All solutions were changed twice daily to prevent the possibility of mineral and oxygen depletion. Embryonic development was monitored daily through the capsule wall using both dissecting and compound microscopes. The time scale used to stage development designates 0 h as the time of oviposition.

For the pulse experiments egg strands were alternately exposed to artificial seawater containing 0 ppm and 8 ppm strontium. The first experiment was designed to show whether any period of the eight to ten day embryonic phase was more sensitive to strontium deprivation and whether the deficits induced by the lack of strontium were reversible. This test began with 16 petri dishes, each containing 10 egg strands. Eight dishes contained basal medium and the remaining eight contained basal medium plus strontium. At the end of Day 1, 24 h after oviposition, five strands from Dish 1 containing basal medium, were transferred to a new dish containing artificial seawater plus strontium (Treatment 1), the remaining five strands served as a control. Likewise, at the end of Day 2, five strands from Dish 2, containing basal medium, were transferred to a new dish containing 8 ppm strontium (Treatment 2). Conversely, egg strands started in artificial seawater plus strontium were transferred to fresh basal medium in a like manner. Thus by the end of Day 8 the experiment included 32 petri dishes, 16 treatments, and their controls (we also ran a natural seawater treatment as a further control). Water was changed twice daily as in the dose response experiments. Figure 1 is a graphical representation of the 16 pulse treatments.

The second pulse experiment was undertaken to confirm the results of the first and to determine the length of the critical period, *i.e.*, we wanted to know whether a short pulse of strontium at the appropriate stage of embryonic development (as indicated by the first experiment) would result in larvae with normal mineralized tissue. This experiment consisted of 11 treatments including controls of basal medium, basal medium plus 8 ppm strontium, and natural seawater. Again, all test media were changed twice daily. Figure 2 represents the various pulse treatments in this experiment. Four treatments called for incubating the egg strands (ten per dish) in basal medium throughout the experiment except for a short pulse of artificial seawater containing 8 ppm strontium. Treatment A was pulsed with strontium for 24 h from the beginning of Day 4 (72 h after oviposition). Treatment B was pulsed for 48 h, from Day 4 to the beginning of Day 6. Treatment C was pulsed with strontium for 24 h from the beginning of Day 5. Treatment H followed the regimen as outlined for Treatment A except natural seawater was used in place of artificial seawater containing 8 ppm strontium.

Conversely, two other treatments required the strands to be incubated in artificial seawater with strontium and pulsed with basal medium at the beginning of Day 4, Treatment D for 24 h, and Treatment E for 48 h. Similarly the last two treatments, F and G, were incubated in natural seawater and pulsed with basal medium at the beginning of Day 4 for 24 and 48 h, respectively.

X-ray Energy Dispersive Spectroscopy (EDS) was used for the qualitative chemical analysis of embryonic shells and statoliths. All specimens were 7- to 8-day-old embryos that had been quick-frozen in liquid nitrogen, while still encapsulated within the egg strand, and stored at −80°C. Two different methods were used for preparing sample material. One procedure included thawing the frozen strands in artificial seawater (+8 ppm strontium) before dissecting for mineralized tissues. Excised stones and shells were then placed in a large drop of distilled water on Formvar-coated 200 hexagonal mesh nickel grids, rinsed by wicking away water, evaporated to dryness, and carbon-coated. In a second procedure we placed the frozen strand in 100% ethanol for five minutes before transferring to a spot dish filled with hexamethyldisilazane (HMDS). After an additional five minutes, the strand was removed from the HMDS, air dried, and transferred to an aluminum stub with double-sided sticky tape. Strands were then broken open with forceps and larvae were arranged on the tape. This material was then carbon-coated.

Analyses were performed on either a Philips-400T transmission electron microscope equipped with STEM and an EDAX spectrometer or a JEOL JSM-840 scanning electron microscope equipped with a Tracor Northern X-ray spectrometer. Shells and stones were examined at 15–40 kV for 500–1000 live seconds. Light micrographs of freshly narcotized material were taken with a Zeiss Photomicroscope III, equipped with polarizing optics.

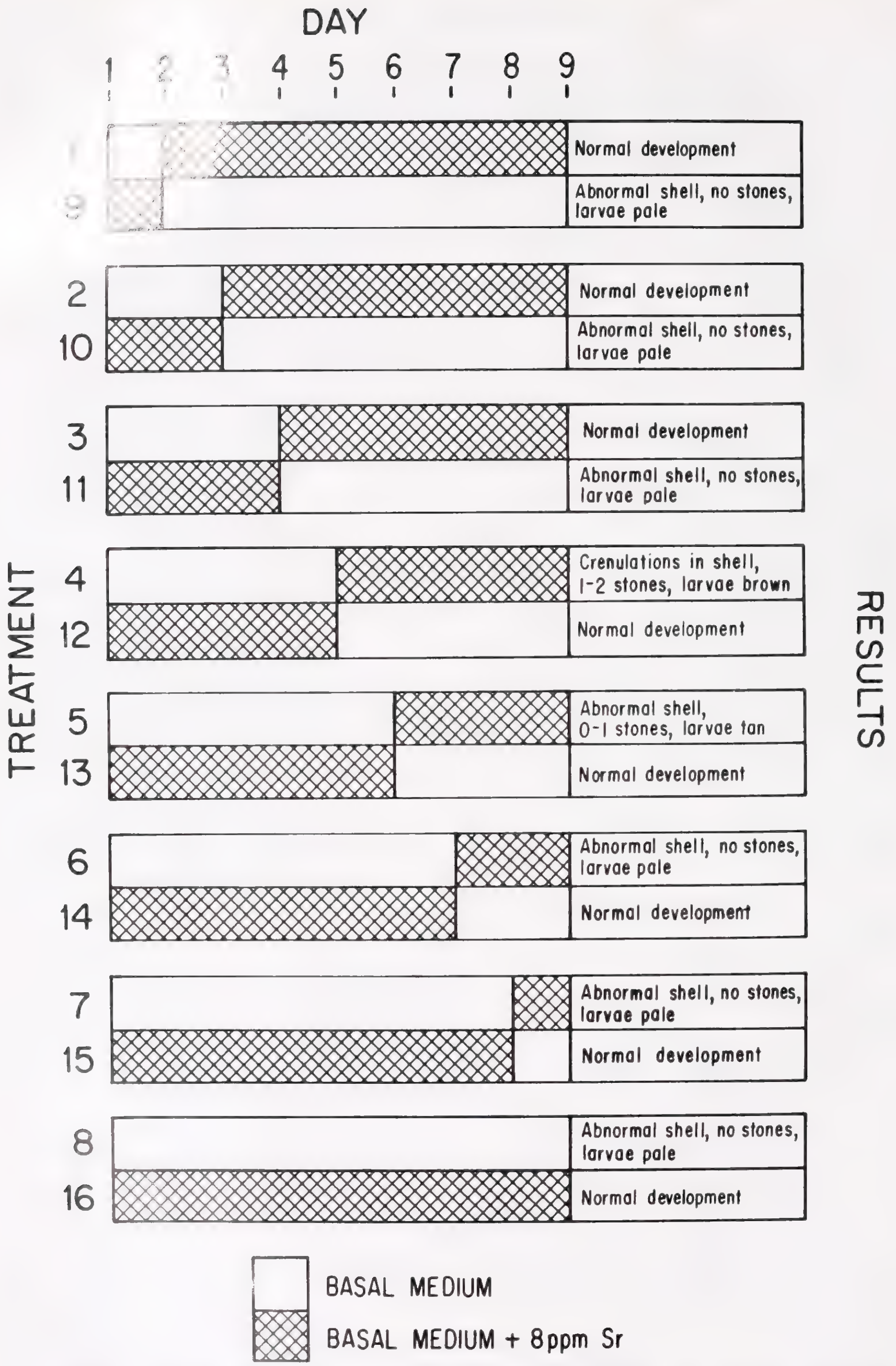

FIGURE 1. Graphical representation and results of sixteen pulse treatments from first pulse experiment. All treatments extended through eight days of embryonic development. Each petri dish generated its own control because 5 of 10 egg strands remained in initial conditions. A natural seawater control is not shown.

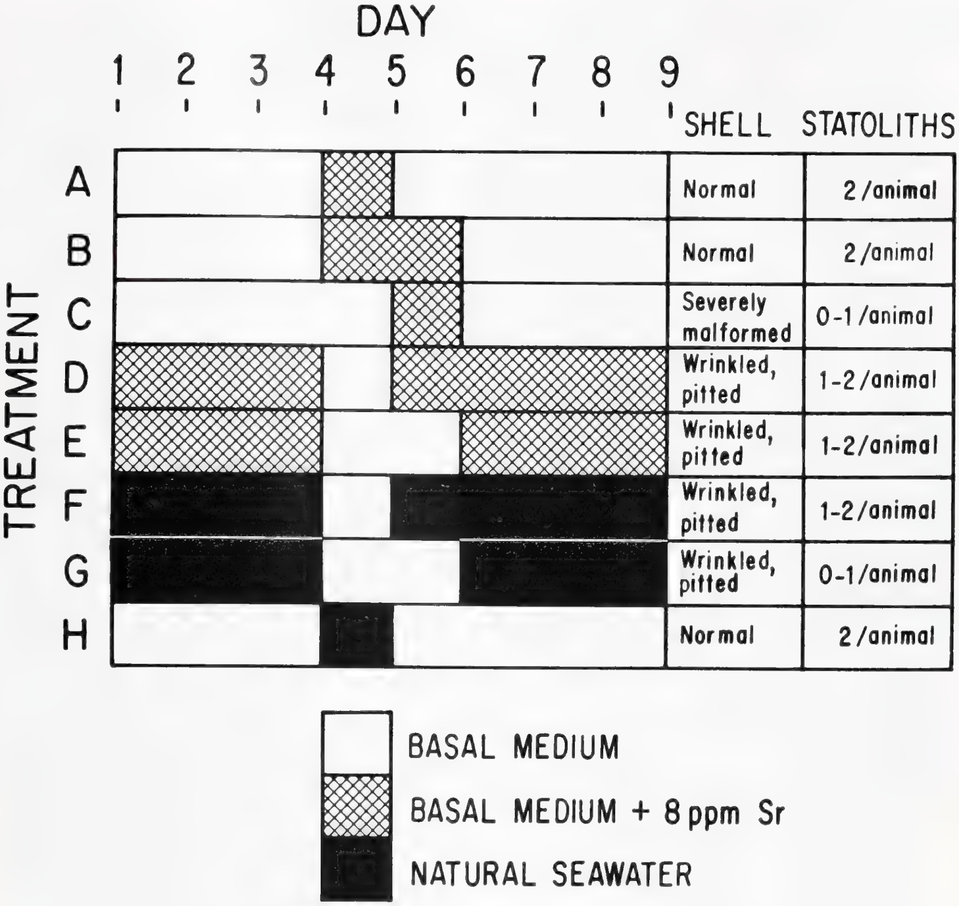
DAY
1 2 3 4 5 6 7 8 9
TREATMENT
SHELL STATOLITHS
A Normal 2/animal
B Normal 2/animal
C Severely malformed 0-1/animal
D Wrinkled, pitted 1-2/animal
E Wrinkled, pitted 1-2/animal
F Wrinkled, pitted 1-2/animal
G Wrinkled, pitted 0-1/animal
H Normal 2/animal
BASAL MEDIUM
BASAL MEDIUM + 8 ppm Sr
NATURAL SEAWATER

FIGURE 2. Graphical representation and results of treatments from second pulse experiment. All treatments extended through eight days of embryonic development. Controls of basal medium, basal medium plus 8 ppm strontium, and natural seawater are not shown.

Finally, to test the effect of strontium on another mollusc, preliminary experiments were performed on the nudibranch *H. crassicornis.* Embryonic development in this animal is very similar to that of *A. californica.* Fertilized egg strands are deposited by the hermaphroditic adult, and during this 8- to 10-day embryonic period, the shell and statoliths are formed. Single egg strings (about 2 cm in length) were placed in 90 cm × 50 cm crystallizing dishes and filled with 200 mls of test medium (basal medium, basal medium plus 8 ppm strontium, or natural seawater). Cultures were incubated at the optimal temperature of 14°C (Donna McPhie, pers. comm.), and test solutions were changed once daily.

RESULTS

Dose response experiments

Egg strands of *A. californica,* incubated in artificial seawater without strontium, produced larvae with deformed shells and statocysts that lacked the large single statolith granule. Soft tissue appeared normal; microscopic observations (82×) made throughout development indicated no differences, morphologically or in the time course of development, from the natural seawater controls. Shell and statolith formation were sensitive to concentration differences of one part per million strontium in artificial seawater treatments (Figs. 3, 4). Differences between embryos developing in media with the full complement of strontium (natural seawater and basal medium containing 8 ppm added strontium) and those organisms cultured in the absence of this element first appeared on Day 5 (104 h after oviposition). In the strontium-free artificial seawater, the shell cap was irregular in shape and the single statolith granule was missing from both statocysts. The appearance of the velum and foot (soft tissues) were indistinguishable from the natural seawater controls. Even the empty statocyst cavities

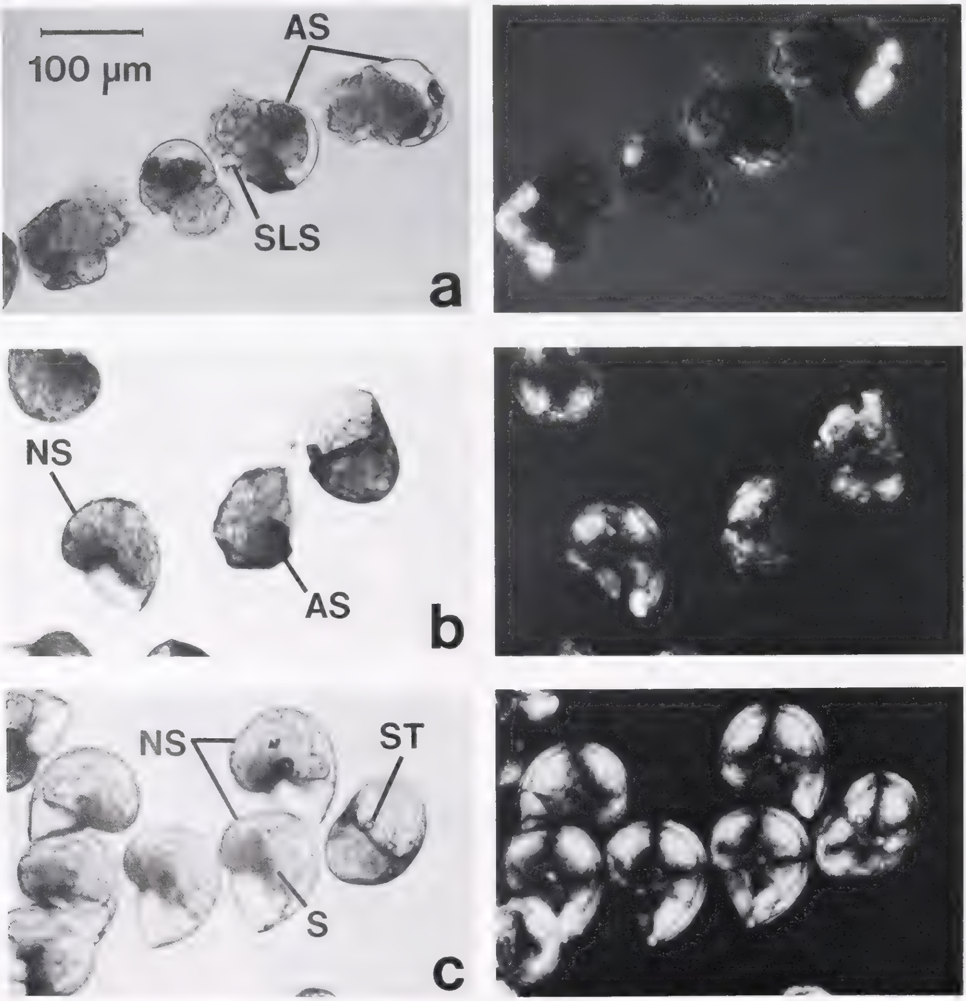

FIGURE 3. Bright field and polarized light micrographs of hatched larvae incubated in artificial seawater during embryonic development at three different strontium concentrations: (a) 1 ppm; (b) 3 ppm; and (c) 7 ppm. Abbreviations: (AS) abnormal shell; (NS) normal shell; (S) statocyst; (SLS) statocyst lacking statolith granule; (ST) statolith granule.

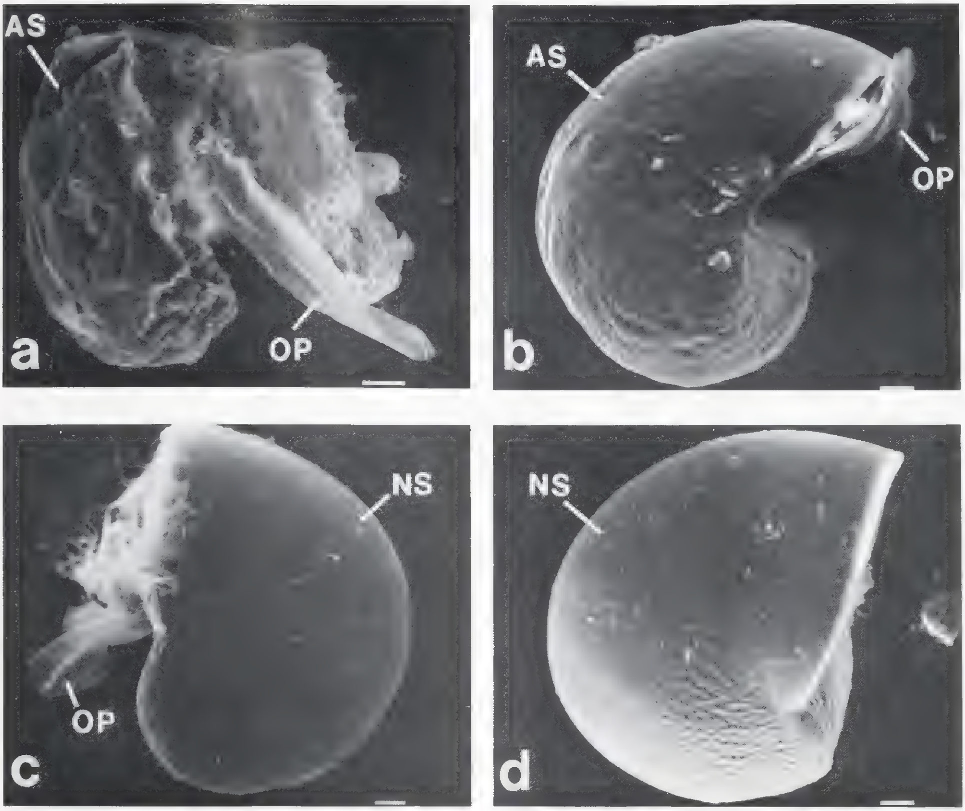

FIGURE 4. Scanning electron micrographs of 8-day-old *Aplysia californica* embryos incubated in artificial seawater at different strontium concentrations: (a) 0 ppm; (b) 3 ppm; (c) 8 ppm; and (d) natural seawater. Abbreviations: (AS) abnormal shell; (NS) normal shell; (OP) operculum. (Scale = 10 μm.)

appeared normal except for the absence of the stones. In the presence of strontium (8 ppm) the shell cap was well defined and the statoliths could be easily seen.

These differences became more pronounced by the end of Day 5 (118 h). Embryos cultured without added strontium had deformed shells that appeared wrinkled, pitted, and were only weakly birefringent when observed under polarized light. The soft tissue had also retracted from the malformed shell.

Another difference noted on Day 5, that became more apparent as development progressed, was the color of the embryonic shell. Darkening of this shell, a brown coloration most evident along the lip and in the whorl, seemed proportional to the amount of strontium in the medium up to 4 ppm. Freshly laid egg strands appear yellow, the color of the fertilized egg visible through the transparent cordon. Those strands cultured in media with 4 ppm or greater added strontium, including natural seawater, appeared to darken from their initial bright yellow color to the normal dark brown observed at the time of hatching. The color of the embryonic shells maintained in the presence of lower concentrations of strontium ranged from a light straw yellow to a darker yellow-tan as the strontium was increased. The empty cordon left behind upon hatching was transparent and colorless after all treatments, further indicating that the color difference was due to color changes in the larvae and not the jelly itself.

All larvae hatched from the egg strands between the normal 200–245 h (8–10 days) after oviposition. Veligers from cultures containing 4 ppm or greater added strontium were vigorous swimmers and indistinguishable in their morphological appearance [see Figs. 3(c) and 4(c, d)] and behavior from those cultured in natural seawater. Larvae incubated in artificial seawater containing 0 and 1 ppm added strontium during embryonic development showed the anomalies described above [see Figs. 3(a) and 4(a)]. Also, these larvae moved in an unusually short, rapid, saltatory manner or swam in tight spinning circles; many remained on the bottom of the petri dish, moving along the surface by rocking back and forth on their deformed shells.

A gradation of these effects could be discerned in cultures containing concentrations of 2 and 3 ppm added strontium. In these cultures the abnormalities in shell shape were not as marked and the shell material was qualitatively more birefringent under polarized light [Figs. 3(b) and 4(b)]. Statoliths were absent in organisms grown in the medium with 2 ppm added strontium, but larvae from the medium with 3 ppm added strontium displayed various degrees of the deficiency. We observed animals with no statoliths and others with one or both stones. The shell deformities also varied but as in the 2 ppm treatment, were not as severe as those obtained in the 0 ppm and 1 ppm regimens. The subtleties of this animal's sensitivity to the differences in strontium levels in the external medium can be seen in the scanning electron micrographs of Figure 4.

The anomalies produced in strontium-free seawater and the other dose treatments were reproducible and affected all organisms in the test samples. Strontium bioassays were performed throughout the year on numerous egg masses from a variety of adults. Egg strands incubated in artificial seawater without added strontium always resulted in larvae with the abnormalities described. Different chemical modifications of the artificial seawater failed to affect embryonic development. For example, the elimination of sodium bromide (another minor constituent of natural seawater) instead of strontium chloride, had no detrimental effect on the animals. Also, the use of the basal medium plus additional salts, a modification of Instant Ocean® (see Materials and Methods), did not compensate for the lack of strontium, and none of these additions proved essential for normal embryonic development.

Pulse experiments

Both pulse experiments indicated the critical period for the strontium requirement of *A. californica* began at Day 4 (72 h after oviposition) and lasted no longer than 24 h (see Figs. 1, 2). In the first pulse experiment (Fig. 1) treatments that produced animals with normal mineralized tissue had only one commonality, embryos were exposed to strontium from Day 4 to Day 5. For example, this 24-h period of exposure to strontium is the only common feature between Treatments 3 and 12, but both regimens produced normal larvae. In contrast, Treatments 11 and 12 are identical except for this critical period, yet larvae from the former (exposure to strontium through *Day 3*) resembled those of the basal medium controls, whereas animals from the latter (exposure to strontium through *Day 4*) were normal.

The results of the second pulse experiment gave a more dramatic demonstration of this critical period. Figure 2 shows that animals exposed to strontium only during this 24-h period (Treatment A) were normal. These larvae had two statoliths and their shells were well-formed and devoid of crenulations. A comparison of the scanning electron micrographs in Figures 4(c, d) and 5(a) demonstrates that larvae from Treatment A were indistinguishable from animals reared in artificial seawater plus 8 ppm strontium or natural seawater throughout embryonic development. Identical results

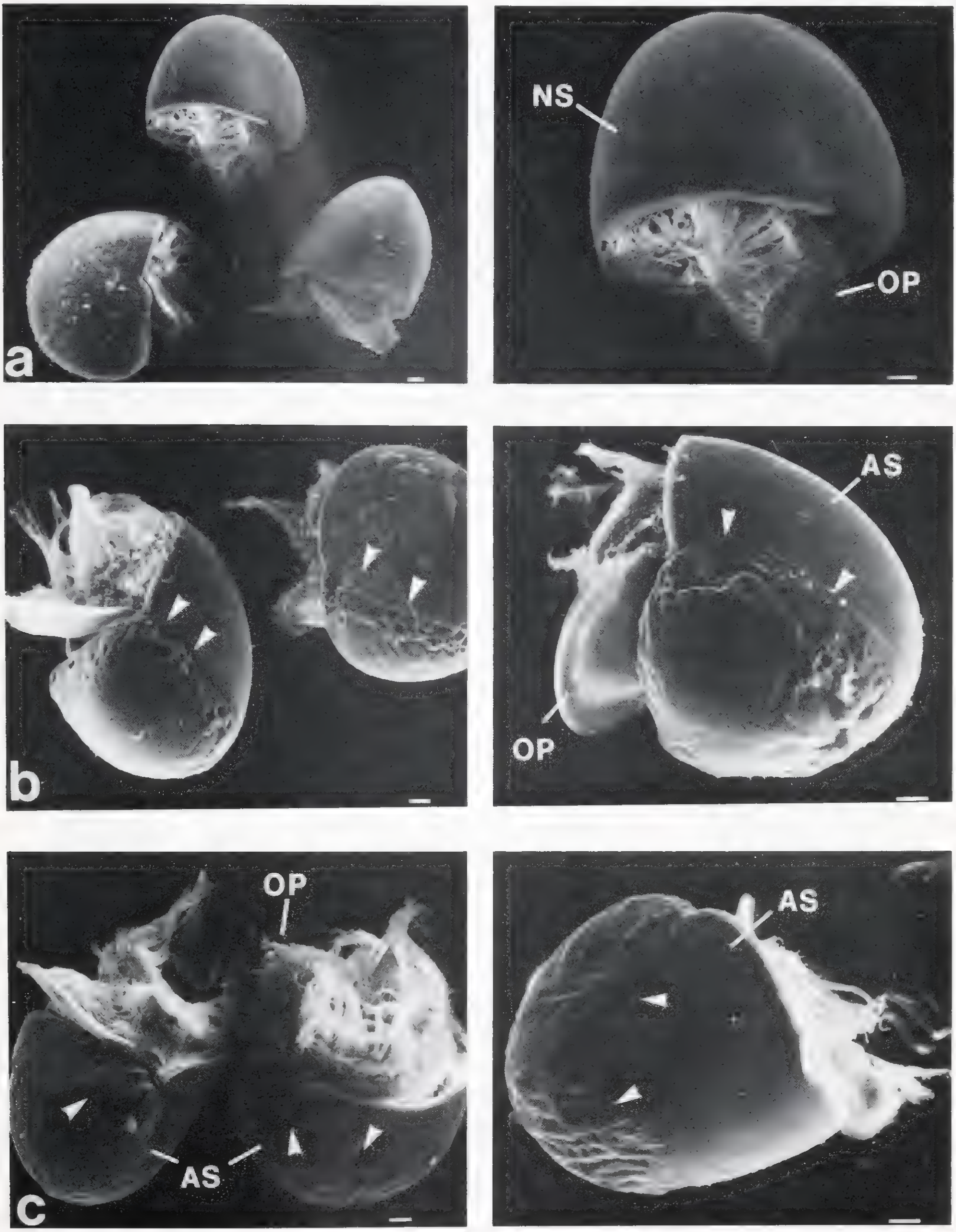

FIGURE 5. Scanning electron micrographs of 8-day-old *Aplysia californica* embryos from second pulse experiment: (a) Treatment A, embryos exposed to 8 ppm strontium for 24 h, Day 4 to Day 5; (b) Treatment D, embryos exposed to 8 ppm strontium throughout development except 0 ppm for 24-h period between Day 4 to 5; (c) Treatment G, embryos exposed to natural seawater throughout development except 0 ppm strontium for 48 h between Days 4 and 6. Abbreviations: (AS) abnormal shell; (NS) normal shell; (OP) operculum; arrows indicate areas of shell deformities. (Scale = 10 μm.)

were obtained with larvae reared in basal medium and exposed to natural seawater during the critical period (Treatment H, Figure 2).

Larvae that had been pulsed with strontium for the 24 h immediately following the critical period, *i.e.*, from Day 5 to Day 6 (Treatment C, Figure 2), were abnormal. These larvae had only one stone when stones were present at all and severely malformed shells. Also the color of the embryonic shells in this treatment was a very light brown whereas the color of the shells in Treatments A and H were indistinguishable from natural seawater or artificial seawater plus strontium controls. In fact, Treatment C was the only regimen to show abnormal shell coloration in the second pulse experiment.

Figure 2 also shows that embryos cultured in media with strontium (artificial seawater plus 8 ppm strontium or natural seawater) and pulsed with basal medium during the critical period resulted in larvae with deficits. Embryos from Treatment D were pulsed with basal medium from Day 4 to Day 5 and grown in artificial seawater with strontium throughout the rest of the embryonic phase. Many animals from this regimen were missing one statolith and the shells were wrinkled and pitted. Scanning electron micrographs [Fig. 5(b)] show the shell abnormalities to be localized in the whorl, that part of the shell formed first. Organisms from Treatment G were pulsed with basal medium for 48 h (Day 4 to Day 6) but otherwise incubated in natural seawater (Fig. 2). Abnormalities were more severe, normal statolith formation appeared to be completely absent with 0–1 stone per animal (a few were found with two small malformed statoliths). The shell defects were more extensive than in Treatment D though again were most evident in the whorl [see Figure 5(b, c)].

The pulse experiments also revealed that deficits induced by strontium deprivation were only marginally reversible. The severity of the abnormalities progressively worsened as exposure to strontium was postponed past the critical window. Exposure before the critical period had no effect on statolith or shell formation. Treatments 9, 10, and 11 from Figure 1 indicate that exposure to strontium before Day 4 did nothing to aid mineralization. All larvae from these regimens resembled those organisms from basal medium controls, having severe shell abnormalities and no statoliths.

Treatments 4 and 5 (Fig. 1) demonstrated that exposure to strontium soon after the critical period *and* extending throughout the remainder of the embryonic period resulted in larvae with less severe abnormalities than those obtained in 0 ppm strontium controls. Larvae from Treatment 4 (exposure to strontium from Day 5) resembled those observed in Treatment D from the second pulse experiment [see Figs. 2, 5(b)]. Crenulations were limited to the shell whorl and a number of larvae were missing one stone. The abnormalities were more severe in Treatment 5 (exposure to strontium from Day 6, see Fig. 1). The vast majority of these larvae lacked stones and the shell margins were more irregular as compared to those obtained in Treatment 4. Finally animals exposed to strontium only after Day 6 (Treatments 6, 7, and 8) resembled those cultured in basal medium throughout the entire experiment.

EDS indicated that mineralized tissues (statoliths and shells) of embryos from all experimental treatments were primarily calcium (as $CaCO_3$). A weak strontium signal could be detected only in treatments that produced normal larvae (*e.g.*, 5 ppm and 8 ppm added strontium). The strontium content of these materials was less than 1% (see Fig. 6), and we were unable to determine if concentrations of shells or stones varied with medium levels. We are now pursuing more quantitative chemical analyses through the use of graphite furnace atomic absorption spectroscopy.

H. crassicornis *experiments*

Preliminary experiments with the nudibranch *H. crassicornis* showed that this animal also required the presence of strontium in defined media for embryonic shell

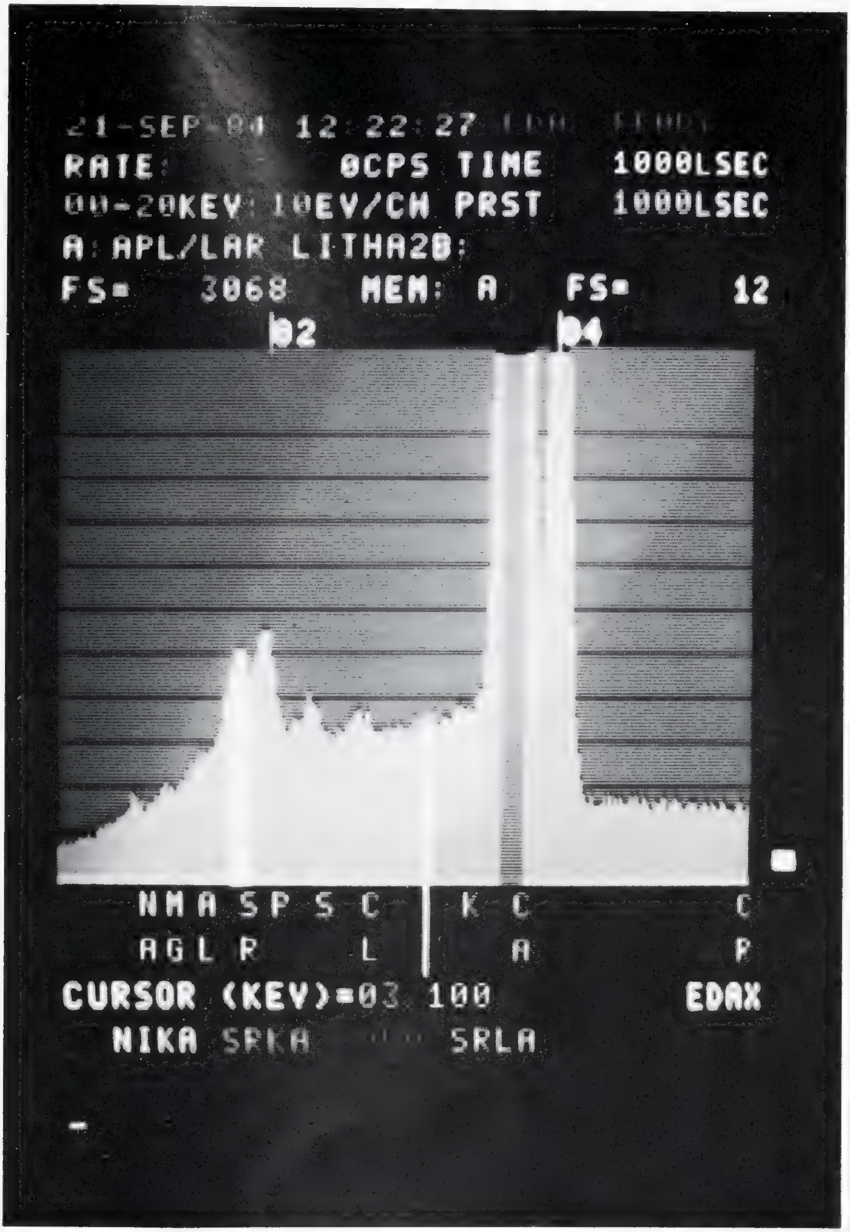

FIGURE 6. Portion of an expanded X-ray energy spectrum (EDS) of a representative statolith from *Aplysia californica* embryo reared in artificial seawater containing 8 ppm strontium. Spectrum obtained with STEM and EDAX spectrometer (20 kV; 1000 live seconds). Data indicates calcium: strontium ratio within the stone approximates that of seawater, *i.e.,* 100:1.

and statolith formation and that the absence of this element induced nearly identical anomalies as described for *A. californica.* Figure 7 shows that deficits appeared to be localized to the mineralized tissue, and we noticed that deformed larvae swam erratically

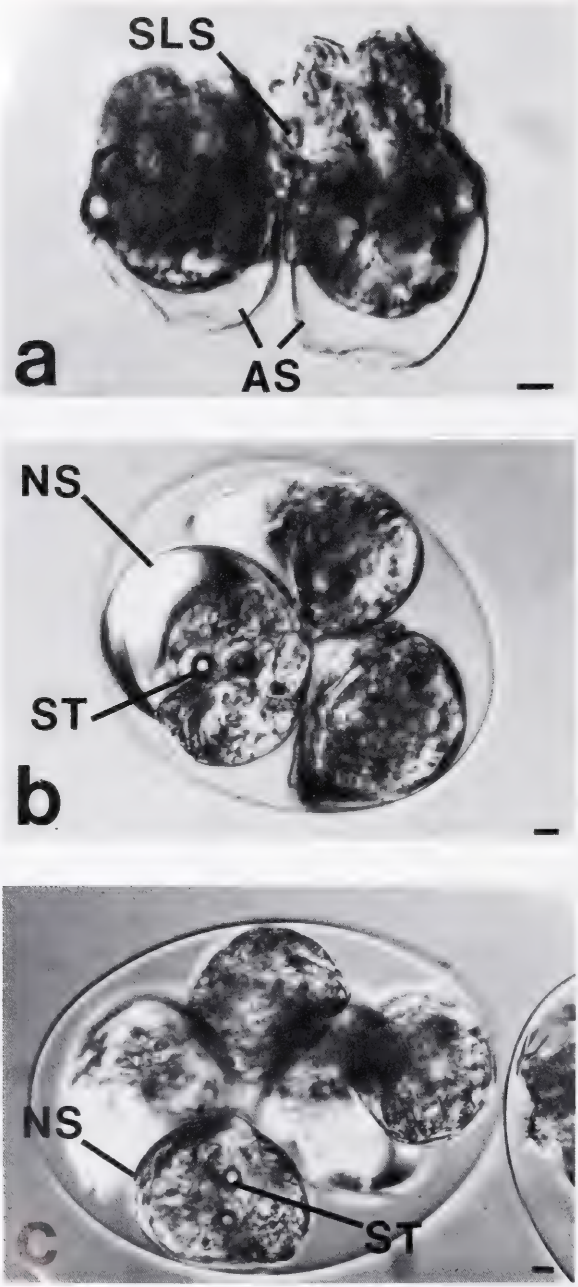

FIGURE 7. Bright field micrographs of 8-day-old *Hermissenda crassicornis* embryos incubated in media with different strontium concentrations: (a) 0 ppm; (b) 8 ppm; and (c) natural seawater. Abbreviations: (AS) abnormal shell; (NS) normal shell; (SLS) statocyst lacking statolith granule; (ST) statolith granule. (Scale = 10 μm.) Note, animals in (b) and (c) are still in egg capsules.

through the water column. The color of the embryonic shell does not normally change during development in this species, and we found that no color changes of any kind were induced by the presence or absence of strontium in the medium.

DISCUSSION

Our study suggests a biological requirement for strontium and indicates a physiological role for this element. Until now strontium has been considered a minor contaminent of both living and fossil tissue (Kinsman, 1969; Riley and Segar, 1970; Binyon, 1978) or as a substitute or tracer for calcium in biochemical studies (Baker and Singh, 1982; Simkiss, 1983). The lack of interest in strontium as a biologically important element *per se* is not surprising since the only organisms with an obvious requirement are the marine protozoa *Acantharia* spp., which possess a test composed of strontium sulfate. Little else is known about this unique exoskeleton since ontogenetic studies establishing specific chemical requirements or the molecular mechanism for its formation have not been reported (see Anderson, 1981).

It appears that strontium is essential to the mineralization process in embryonic *A. californica.* To date, no one has directly addressed the possible role of trace metals in shell deposition, and there has been little attempt at a synthesis of the vast amount of information on factors that affect the trace metal content of shells and the mineralization process itself (see Wilbur, 1972; Simkiss, 1976; Wilbur and Saleuddin, 1983). The strontium content of mollusc shells is low, but differences in shell strontium content usually have been interpreted as a measure of an animal's ability to exclude it from the calcium carbonate-organic matrix (Dodd, 1967). In fact, it has been suggested that the higher the phyletic level of the organism the greater its ability to exclude "foreign ions," such as strontium and magnesium, from the skeleton (Dodd, 1967).

Our results suggest that embryos of *A. californica* take up strontium from seawater and use it for structural and sensory development. Deprivation of strontium during this early stage results in an animal with morphological and behavioral deficits. The morphological deficits include the malformed shell and its abnormal color development. Darkening of the larvae within the egg strand, from yellow to brown, and its dependence on strontium concentration reflects the extent of shell mineralization occurring within the egg capsules. Kriegstein (1977) also noted that the lip of the shell in newly hatched veligers is characteristically golden-brown. Therefore, a possible explanation is that the dark coloration arises from the organic material or matrix (Wilbur, 1972) laid down during mineralization which is visible through the transparent cordon. Because shell formation is dependent on the strontium concentration of the seawater, so then is the apparent color development of the larvae within the egg strand. Finally, the other deficit is the absence of the statoliths. The spinning behavior, induced by strontium deprivation, can be explained by the absence of statoliths whose formation appears to require this element at the beginning of Day 4 (72 h after oviposition). The invertebrate statocysts are balance receptors (Coggeshall, 1969; for review see Markl, 1974) and provide a directional sense through the interaction of the stone with the sensory cilia of the hair cells (Colmers, 1977; Budelmann, 1979; Stommel *et al.,* 1980). In *Aplysia* spp. they coordinate locomotion and swimming (Kandel, 1979), and we find that affected larvae move erratically upon hatching.

Although structural defects of the shell may contribute to spinning, the lack of statoliths and subsequent loss of sensory input is sufficient to explain the behavioral disorder. Our experience indicates that the deformed shell does little to inhibit movement. For example, in our culture work, we have observed larvae with malformed shells and fully developed statoliths that swim normally; also, larvae that have left their shells do not spin. A similar spinning syndrome has been reported in a number of cephalopod larvae which have only rudimentary shells (Colmers *et al.*, 1984). "Spinner" cephalopod larvae (three species of squid and three of octopus) swim erratically upon hatching in recirculating systems of artificial seawater. The only morphological deficit in these animals is the missing statolith granule in both statocysts. Another example of abnormal statolith development affecting behavior is that reported by Crow and Harrigan (1979) in the nudibranch *H. crassicornis.* Animals raised in the laboratory developed statocysts with a single statoconium as compared to normal statocyst cavities filled with many statoconia. The authors suggested that this morphological difference between laboratory-reared and field-collected adult *H. crassicornis* explained some of the reduced variability in the response to light of the laboratory-reared animals.

The required presence of strontium in artificial seawater during embryonic development and the distinct consequences of its absence is not limited to *A. californica.* In fact, each mollusc tested under the conditions described in this paper has demonstrated a requirement for this element during embryonic mineralization with nearly identical symptoms of the deficiency. As described above, larva of the nudibranch *H. crassicornis* have malformed shells and lack statoliths when incubated in basal medium as embryos. Also, collaborative studies with Roger Hanlon of the Marine Biomedical Institute in Galveston have demonstrated that strontium is required for statolith formation in two species of squid, *Loligo pealei* Lesueur, 1821, and *L. vulgaris* Lamarck, 1799. Embryos incubated in basal medium became squid larvae with soft tissue that appeared normal but with empty statocyst cavities, *i.e.*, "spinners." The addition of strontium to the incubation medium resulted in larvae with normal stones (Hanlon *et al.*, in prep.).

Finally, we have determined that the shipworm *Bankia gouldi* (Bartsch, 1908) (Class Bivalvia) also appears to require strontium. These animals are oviporous broadcast spawners that undergo embryonic development in the 24-h period immediately following fertilization. During this stage of development, the hinged prodisoconch I is formed; the statoliths, however, develop about 18 days later during the larval phase. The absence of strontium from the incubation medium during the first 24 h prevented shell formation, while again the soft tissues appeared to proceed through normal development. The shell-less animals were vigorous swimmers. The addition of strontium resulted in normal larvae (Gallager and Bidwell, in prep.).

The molecular mechanism for producing the behavioral and structural deficits observed in *A. californica* is not yet known, but the pathogenesis seems clear. The shell and stones are primarily calcium carbonate (Coggeshall, 1969) a crystal that does not require strontium for its formation *in vitro.* Yet crystal formation *in vivo* is very sensitive to the strontium chemistry of the external medium. Pulse and dose response experiments demonstrate that the shell crystal is a sensitive visual record of the chemical history with respect to the amount of strontium in the medium and the time of exposure. Also it appears that this organism has little ability to store this element before the critical period of Day 4, the onset of mineralization.

How then is strontium used in the formation of skeletal carbonate and what is the significance of the critical window of exposure? Perhaps this element is a cofactor of an enzyme involved in initiating mineralization during the formation of the shell cap and the single statoconium, though to date, no strontium metalloenzyme has been characterized. Three enzymes that have been implicated in the biomineralization process are carbonic anhydrase, alkaline phosphatase, and the ATPases (Simkiss, 1976; Wilbur and Saleuddin, 1983). Alkaline phosphatase is an attractive candidate. Though little is known about its role in mineralization, it has been suggested that it may be involved in removing crystal poisons (Simkiss, 1964) or synthesizing matrix material (Bradfield, 1950; Kroon, 1952). The metallo-chemistry of this large and ubiquitous family of enzymes is suggestive of a place for strontium. It is generally agreed that zinc and an alkaline earth metal such as magnesium or calcium are an integral part of most of these proteins (McComb *et al.*, 1979). Also, strontium can partially restore the activity of a calcium containing alkaline phosphatase from *Micrococcus sodenensis* (Glew and Heath, 1971). The action of strontium in *A. californica* is specific since the large amounts of calcium and magnesium present in the medium are unable to substitute for the small amount of strontium essential for mineralization. Also, the presence of a variety of trace metals, boric acid, and inorganic phosphate cannot compensate for the lack of strontium. In fact, we find that the simple basal medium plus strontium has been sufficient for rearing *A. californica* through an F_6 generation.

Our results have important implications for a variety of disciplines including molluscan physiology, neurobiology, and paleoecology. Until now strontium has been viewed in the context of a minor contaminent of mineralized tissue, an element with a similar chemistry to calcium and thus a convenient probe for shell deposition, not as a requirement to a vital pathway of the process itself. Also, paleoecologists have long recognized that if strontium is to be used as an indicator of ancient environments, its physiological and diagenetic activities must be disentangled (Rosenberg, 1980). Until now, however, any understanding of strontium's biological importance has not progressed beyond reporting contents of this element in living and fossil shell material (Rosenberg, 1980). Finally, strontium appears to be essential for the normal development of behavior in *A. californica, Loligo pealei, L. vulgaris,* and *H. crassicornis* through its effect on formation of statoliths.

ACKNOWLEDGMENTS

We thank Ms. Donna McPhie of the Laboratory of Biophysics, NINCDS-NIH at the Marine Biological Laboratory (MBL) for carrying out the experiments with *Hermissenda crassicornis.* Scott Gallager of the Woods Hole Oceanographic Institution (WHOI) performed the experiments on *Bankia gouldi.* We thank Drs. Roger Hanlon, June Harrigan (MBL), and Judith Capuzzo (WHOI) for helpful discussions. Drs. James H. Schwartz (Columbia University, Howard Hughes Medical Institute) and Carl J. Berg (MBL) critically read the manuscript.

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GROWTH AND SEXUAL MATURATION OF LABORATORY-CULTURED MONTEREY *BOTRYLLUS SCHLOSSERI*

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ABSTRACT

Growth and sexual maturity were compared among Monterey *Botryllus schlosseri* colonies cultured in the laboratory and in Monterey Marina in two separate studies: Phase I—April to July 1984, a time when *Botryllus* growth is rapid in the field, and Phase II—December 1984 to March 1985, a time when growth is minimal in the field. Laboratory colonies were kept in tanks of standing, aerated filtered seawater at 15°C, 20°C, and 24°C; the seawater was changed daily or on alternate days. Food types were two concentrations of an algal mixture and five concentrations of Liquifry, a commercially available food for filter-feeding marine invertebrates. All algae-fed colonies did poorly. During Phase I, the colonies kept in Monterey Marina grew much faster than those in the laboratory, but they took longer to reach sexual maturity than the Liquifry-fed colonies grown at 20°C and had a lower survivorship. In Phase II, *Botryllus* growth rates and survivorships in seven of the eight laboratory treatments were better than those of marina colonies. These laboratory treatments yielded a substantial proportion of sexually mature colonies, whereas none of the marina colonies produced eggs during Phase II.

INTRODUCTION

The colonial tunicate *Botryllus* has become increasingly important as an experimental animal. It has been the subject of research in developmental biology (Mukai and Watanabe, 1976), genetics (Sabbadin, 1964; Scofield *et al.,* 1982), immunology (Taneda and Watanabe, 1982; Scofield and Nagashima, 1983; Schlumpberger *et al.,* 1984), ecology (Grosberg, 1981, 1982), and physiology (Mackie and Singla, 1983). Research endeavors would be enhanced by the existence of genetically defined, sexually reproductive stocks of *Botryllus* colonies maintained in the laboratory.

Laboratory cultures of Monterey *Botryllus schlosseri* will allow circumvention of seasonal variation in colony availability, growth rate, and sexual reproduction, and will permit the production of defined genetic stock colonies. We attempted to identify optimal culture conditions by comparing the growth rate and onset of egg production among colonies raised at different temperatures and fed different diets. Treatment conditions were partially based on earlier methods applied to laboratory culture of colonial ascidians in the northeastern United States (Berrill, 1937; Milkman, 1967), Italy (Sabbadin, 1960), and Japan (Nakauchi *et al.,* 1979).

Received 3 June 1985; accepted 29 October 1985.

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MATERIALS AND METHODS

Procedural differences between Phases I (April–July 1984) and II (December 1984–March 1985) were primarily due to experiences and results of Phase I.

Animals

Phase I. Monterey *Botryllus sp.* has been classified as *B. schlosseri* by T. Newberry (University of California, Santa Cruz), in collaboration with Y. Saito and H. Boyd (Hopkins Marine Station of Stanford University). Wild-fertilized *Botryllus* colonies were collected from algal substrates attached to floating docks in Monterey Marina (Monterey, California). Each of five colonies containing mature embryos was placed in filtered (5–10 microns) seawater in a transparent 266 ml plastic cup that had been wrapped with black tape. A clear plastic petri dish, covered with a black plastic circle nearly the diameter of the top of the cup, served as a loose-fitting lid. This cup was placed in a light-tight box (66 × 33 × 42 cm), containing a Vita-Lite (Duro-Lite, 15WT8) and equipped with an externally mounted cooling fan (Archer, 10.5 cm diameter) and exhaust port. The box was kept in a 15°C temperature-controlled room. The Vita-Lite and fan were controlled by a timer which was set to approximate the natural daylength. When the cup was placed under the Vita-Lite inside the box, its lid created a shadow on the water surface, while still allowing some light to reach the colony. Upon hatching from the colony, *Botryllus* tadpoles are positively phototactic and negatively geotactic (Grave, 1937), and thus many tadpoles swam upward and metamorphosed, inverted, at the water's surface. When their tails were almost completely resorbed, the oozooids were transferred with a Pasteur pipette to a 7.6 × 5.1 cm glass slide; two oozooids were placed about 2 cm apart in the center of each slide. Progeny from the 5 colonies were distributed equally among the different treatments, with a total of 14 oozooids per treatment. On 4/23/84 (considered as day 0 for Phase I), these colonies were 18–27 days old, depending upon the date of hatching from their respective maternal colony.

Phase II. Tailwrap embryos were dissected from each of nine wild-fertilized Monterey *Botryllus* colonies and placed in 10 ml filtered seawater, containing 40 μl of Penicillin (10,000 U/ml)-Streptomycin (10 mg/ml) (P/S), in individual Poly-Con dishes (Cole-Parmer Instrument Co.; 2 cm high × 4 cm diameter). The dishes were kept closed, at 15°C or at room temperature, with a daily change of filtered seawater-P/S, until hatching occurred. Newly metamorphosed oozooids were carefully peeled from the sides and bottom of the dish with the aid of forceps and dissecting microscope. Each oozooid was placed in the center of a 7.6 × 5.1 cm glass slide. Progeny from the 9 colonies were distributed equally among the different treatments, with a total of 14 oozooids per treatment. On 12/3/84 (day 0 of Phase II), colonies were 18–58 days old, depending upon the date of hatching from their respective maternal colony.

Physical set-up

Phase I. Slides containing colonies to be maintained in the laboratory were placed vertically in adjacent slots of glass staining racks and kept in eight individual glass tanks containing 4 liters of standing filtered seawater and aerated with an airstone. Typically, this allowed for at least 10 ml of filtered seawater per zooid during the experiment (Milkman, 1967). The seawater was changed daily in each tank. The tanks were kept in the 15°C room. Half of the tanks each contained a 50 watt aquarium heater to maintain the water temperature at 20°C. The colonies in all tanks were exposed to 14 hours of indirect light (1.5 microeinsteins/m^2/s), in order to approximate the daylength during the season when most colonies in the Monterey Marina contain eggs and fertilized embryos.

A cohort of colonies on slides was kept in a wooden rack in the Monterey Marina about 60 cm below the water surface, near where wild colonies are found. These slides were placed horizontally in the rack, with colonies on the undersides of the slides.

Phase II. In the 15°C room, slides containing laboratory-maintained colonies were placed in alternate slots of glass staining racks in eight individual glass tanks containing 17 liters of standing filtered seawater and aerated with an airstone. The seawater was changed daily in one of the tanks, but was only changed on alternate days in the remaining seven tanks. Using individual 50 watt aquarium heaters, the water temperature in five of the tanks was maintained at 20°C, and at 24°C for the other three tanks. Lighting conditions were as in Phase I.

A set of colonies was kept in the marina under the same conditions as described for Phase I.

Food

Phase I. Half of the laboratory-maintained colonies were fed once daily with a mixture of the green alga *Dunaliella tertiolecta* (obtained from the University of Texas Algae Collection, Austin, Texas) and the diatom *Phaeodactylum tricornatum* (obtained from Moss Landing Marine Laboratory, Moss Landing, California). *Dunaliella* was grown in Provasoli's Enriched Seawater and *Phaeodactylum* was grown in the same medium without iron (Stein, 1973). Algae suspensions were dispensed to each tank by pipette. Cell concentrations for "1× algae" in the tanks were 2.5×10^4 cells/ml for each algal species. Choices of algae and their concentrations were based on studies by Milkman (1967), Sabbadin (1960), and Nakauchi *et al.* (1979).

The remaining half of the laboratory-maintained colonies were fed once daily with Liquifry marine (Interpet Ltd., Dorking, England), a liquid food for filter-feeding marine invertebrates. Liquifry contains dextrin, pea flour, whole egg, yeast, spinach, and sulphurous acid (preservative). It consists of $\geq$3.5% crude protein, $\geq$1.6% crude fat, and $\leq$1.0% crude fiber. The designation "1× Liquifry" was equivalent to 37.5 μl per liter of a 1:10 dilution of Liquifry in filtered seawater. The Liquifry solution was delivered to each tank by pipette.

Phase II. All laboratory tanks received either 2×, 4×, 8×, or 16× Liquifry once daily (see Phase I).

Growth and colony condition

In Phase I, the initial number of zooids per colony was recorded on 4/23/84 (day 0); the experiment began on 4/26/84 and ended on 7/10/84 (day 78). Experimental conditions for Phase II began on 12/3/84 (day 0) and the initial number of zooids per colony was recorded on this date. Phase II was terminated on 3/14/85 (day 101). All colonies in Phase II were cleaned at least once each week with a small paintbrush. Growth was measured by counting the number of zooids per colony once each week (and once every two weeks at the end of Phase II). In addition, during Phase II growth observations, colony condition was recorded based on morphology of zooids and ampullae and integrity of tunic. A colony's condition was rated as "satisfactory" if the majority of its zooids and ampullae were well-formed (*i.e.*, not shrunken), had vigorous blood circulation, and a firm tunic.

Sexual maturity

In Phases I and II sexual maturity was determined by the presence of newly metamorphosed zooids, unfertilized eggs, or embryos developing inside the zooids (the latter observation sometimes involved dissection of zooids in Phase I). This is a conservative definition of sexual maturity, because *Botryllus* colonies develop testes before they produce eggs (Milkman, 1967; and pers. obs.). However, we decided to focus on the presence of eggs or embryos because of the need for breeding colonies to establish defined genetic stocks. Once a colony was observed to have eggs or embryos, it was automatically scored as being sexually mature in subsequent weeks; this has been our general observation with Monterey *Botryllus*.

Data analysis

Treatment means and standard errors for the number of zooids per colony were computed for each weekly observation, excluding dead colonies. The growth data in both Phases I and II were analyzed nonparametrically according to Steel and Torrie (1960) or Sokal and Rohlf (1981). Nonparametric tests were used because the heterogeneity of treatment variances could not be alleviated by standard transformations of the data, and the data had distinctly non-normal distributions. (Since tests on the same colonies at different dates in the time series are not strictly independent, the probabilities in such tests should be interpreted with some caution.) Where the data consisted of blocks determined by the maternal parent, the Wilcoxon signed rank test, which is a nonparametric equivalent of the *t*-test for paired data, was used for comparison of two treatments. The Friedman's test, which is analogous to a randomized block analysis of variance, was used for the comparison of more than two treatments. With significant Friedman's tests ($P < 0.05$), treatment means were compared with Wilcoxon-Wilcox tests.

In Phase II, four additional variables were analyzed as above: (1) Maximum size (number of zooids per colony) was used as an index of growth in a treatment; (2) The number of days in the experiment required to reach 50% of maximum size was used as an index of growth rate; (3) The number of days in the experiment required to reach sexual maturity was used to investigate how rapidly reproductive colonies could be obtained; (4) Colony size at sexual maturity was analyzed to determine which treatments yielded the largest colonies at the onset of sexual maturity. Where colony mortality, or the failure of some colonies to achieve sexual maturity, resulted in a non-balanced experimental design, comparisons involving two treatments were done with the Wilcoxon rank sum test, and comparisons involving more than two treatments were made with Kruskal-Wallis tests. When the Kruskal-Wallis test was significant ($P < 0.05$), further comparisons between pairs of treatments were made with the Wilcoxon rank sum test. The four variables were also analyzed to compare young colonies (18–32 days old at day 0) to old colonies (46–58 days old at day 0) in Phase II to determine if colony age affected the responses to the treatments.

Experimental design

The experimental designs of Phases I and II are shown in Table I.

RESULTS

Phase I: Growth of Liquifry-fed colonies was superior to that of colonies fed algae

The *Botryllus* colonies that were fed a mixture of *Dunaliella* and *Phaeodactylum* at 15°C and 20°C had a low growth rate (Fig. 1A). *Dunaliella* tended to stick to, and

TABLE I

Experimental designs

PHASE I (April–July 1984)		
Laboratory treatments:	*15°C*	*20°C*
	1× algae[a]	1× algae
	2× algae	2× algae
	1× Liquifry[a]	1× Liquifry
	2× Liquifry	2× Liquifry
Field treatment: Monterey Marina		
PHASE II (December 1984–March 1985)[b]		
Laboratory treatments:	*20°C*	*24°C*
	2× Liquifry (daily)[c]	—
	2× Liquifry (alternate)[c]	—
	4× Liquifry	4× Liquifry
	8× Liquifry	8× Liquifry
	16× Liquifry	16× Liquifry
Field treatment: Monterey Marina		

[a] For details of algae and Liquifry concentrations, see Food section (above).

[b] In the text, Phase II treatments will be referred to as follows: 2×,20°C (daily); 2×,20°C (alternate); 4×,20°C; . . . 4×,24°C; and so forth.

[c] Filtered seawater was changed daily in the first 20°C treatment in Phase II, thus following the same procedure as in Phase I. Filtered seawater was changed on alternate days in the remaining seven laboratory treatments in Phase II.

presumably grow on, the animals. The algal treatments were terminated on day 57, when survivorship of colonies in the 1× algae,20°C treatment was about 70%, and survivorships for the other three treatments were below 30%. By this date, the surviving colonies had small poorly developed zooids and sluggish blood circulation.

The animals fed Liquifry at 15°C and 20°C grew larger and were healthier than those fed algae (Fig. 1B). During the first 20 days, all colonies remained small, including those in the marina. By day 50, the colonies grown at 20°C were significantly larger, as a group, than the colonies grown at 15°C (Friedman's test: Chi-square = 35.9, d.f. = 3, $P < 0.01$). However, in the final stages of the study, the growth curves for animals kept at 20°C tended to level off. By day 78, only the 1× and 2× Liquifry treatments at 15°C were significantly different from one another (Wilcoxon signed rank test: Z = 3.2, $P < 0.01$). The animals fed 2× Liquifry at 15°C had been growing rapidly in this final phase and by day 78 the mean colony size for this treatment was not significantly different from that for animals fed 1× Liquifry at 20°C (Wilcoxon signed rank test: Z = 0.3, $P = 0.40$). On day 78, survivorships for all four Liquifry treatments ranged from 93% to 100%.

From day 22 to the end of the study, the mean size of colonies growing in Monterey Marina was larger than those of the colonies grown in the laboratory (Figs. 1A, B). The marina colonies approximately doubled in size weekly from day 22 to day 50. Survivorship in the marina was about 80% on day 78.

Phase I: Sexual maturity occurred in laboratory-maintained colonies at 20°C

Sexual maturity occurred in colonies fed Liquifry at 20°C or grown in the marina (Fig. 2). Eggs and/or embryos were first observed in these Liquifry treatments on day

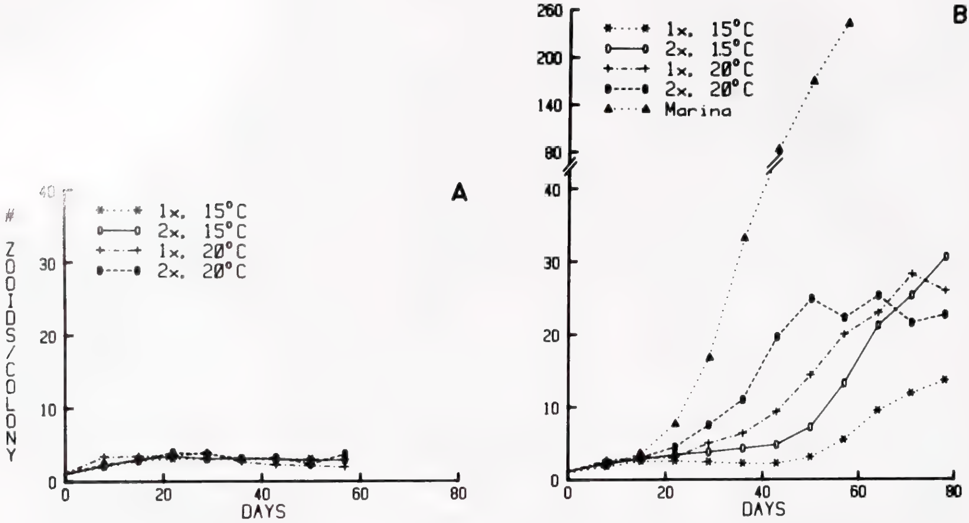

FIGURE 1. *Botryllus* growth curves for Phase I. The ordinate represents mean number of zooids per colony. Day 0 = 4/23/84. Note that 1×, 2×, etc. refer to the food concentrations, and not the frequency of daily feeding. (Zooid counts were not continued for marina colonies after day 57 because the colonies were very large, such that in some cases the growing edges of two colonies on a slide had made contact, sometimes resulting in vascular fusion.) A: Colonies fed 1× or 2× algae at 15°C and 20°C. B: Colonies fed 1× or 2× Liquifry at 15°C and 20°C, or grown in Monterey Marina.

57; by day 78, approximately 70% of the 1× Liquifry colonies and nearly 60% of the 2× Liquifry colonies were sexually mature. In the marina, eggs and/or embryos were first observed on day 50; by day 78, only about 40% of the marina colonies were sexually mature.

Phase II: Healthy, rapidly growing Botryllus *colonies were raised under longterm controlled laboratory conditions*

Although *Botryllus* colonies grow well in Monterey Marina from April to July (Phase I), we suspected that this would not be the case during winter months. To investigate this further, we initiated Phase II culture treatments in December, and chose higher temperature and food levels for the laboratory conditions based on the results from the Phase I experiment. Our major finding was that *Botryllus* colonies raised on a Liquifry diet at 20°C and 24°C in the laboratory grew faster than colonies grown in the marina in winter. In addition to the initial growth increase experienced by all colonies, those grown at the higher food levels at 20°C underwent a second growth rise. As in Phase I, the survivorship of colonies in the marina treatment was lower than those for all laboratory treatments in Phase II.

Before the details of Phase II culture are discussed, the high degree of variability among *Botryllus* colonies with respect to growth should be noted. Individual growth curves for the colonies in each treatment in Phase II are depicted in the Appendix figure. Although the distribution of colonies is matched across treatments with respect to age and maternal origin, it is clear that, even under specified environmental conditions, *Botryllus* colonies as a group do not exhibit readily defined growth patterns. The high variability among colonies within treatments necessitated the use of nonparametric statistics for data anlaysis.

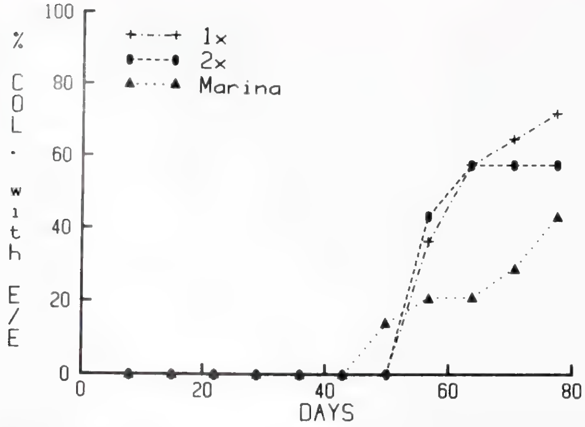

FIGURE 2. Sexual maturity of *Botryllus* colonies in Phase I. The ordinate represents cumulative percentage of colonies with eggs and/or embryos. Day 0 = 4/23/84. Data are for colonies fed 1× or 2× Liquifry at 20°C, and for colonies grown in Monterey Marina.

The effect of frequency of seawater change on growth. In Phase I, filtered seawater was changed daily in all of the laboratory treatments. In Phase II, daily and alternate day seawater change were compared under treatment conditions that yielded the best colony growth in Phase I. The growth curves of colonies fed 2× Liquifry at 20°C receiving daily filtered seawater change [2×,20°C (daily)] and those receiving alternate day filtered seawater change [2×,20°C (alternate)] were similar (Fig. 3A), although the mean sizes for colonies receiving alternate day changes of seawater were significantly higher from day 30 to day 51 of the experiment (Wilcoxon signed rank tests, $P < 0.05$). The survivorships at day 101 for these two treatments were greater than 90%.

The effect of varying food concentration on growth at 20°C. Figure 3B depicts the growth of colonies at 20°C. During the first month of the experiment, the growth rates of all four Liquifry treatments were similar. Notably, a second growth rise occurred initially in the 16× and then in the 8× Liquifry treatment; the 2× (alternate) and 4× treatments did not exhibit this second increase in growth rate (also see Appendix Figs. B–E). There were no significant differences among the four Liquifry treatment means after day 58 due to the high degree of variability among colonies. Survivorships at day 101 for these Liquifry treatments at 20°C were greater than 90%.

The effect of varying food concentration on growth at 24°C. Growth data for the 24°C laboratory colonies are plotted in Figure 3C. By visual inspection, 8× Liquifry gave the best growth, 4× Liquifry yielded the next best results, and the 16× Liquifry treatment was clearly unacceptable for *Botryllus* culture at 24°C; the 16× Liquifry treatment was terminated on day 58, when more than one-third of the colonies had died, and the remaining colonies were in poor condition. During the first month of Phase II, the 4× and 8× Liquifry treatments had similar growth curves; subsequently the curves separated, with 8× Liquifry maintaining a larger mean colony size than 4× Liquifry throughout the remainder of the experiment. However, the weekly 4× and 8× treatment means were never significantly different from one another due to the high variability among colonies, particularly within the 8× treatment (Wilcoxon signed rank tests, $P > 0.05$). At the end of the experiment, the survivorship was 100% for the 4×,24°C treatment and about 80% for the 8×,24°C treatment.

The effect of varying temperature on growth. Mean colony sizes were compared between the 20°C and 24°C treatments having the same food concentration. Mean

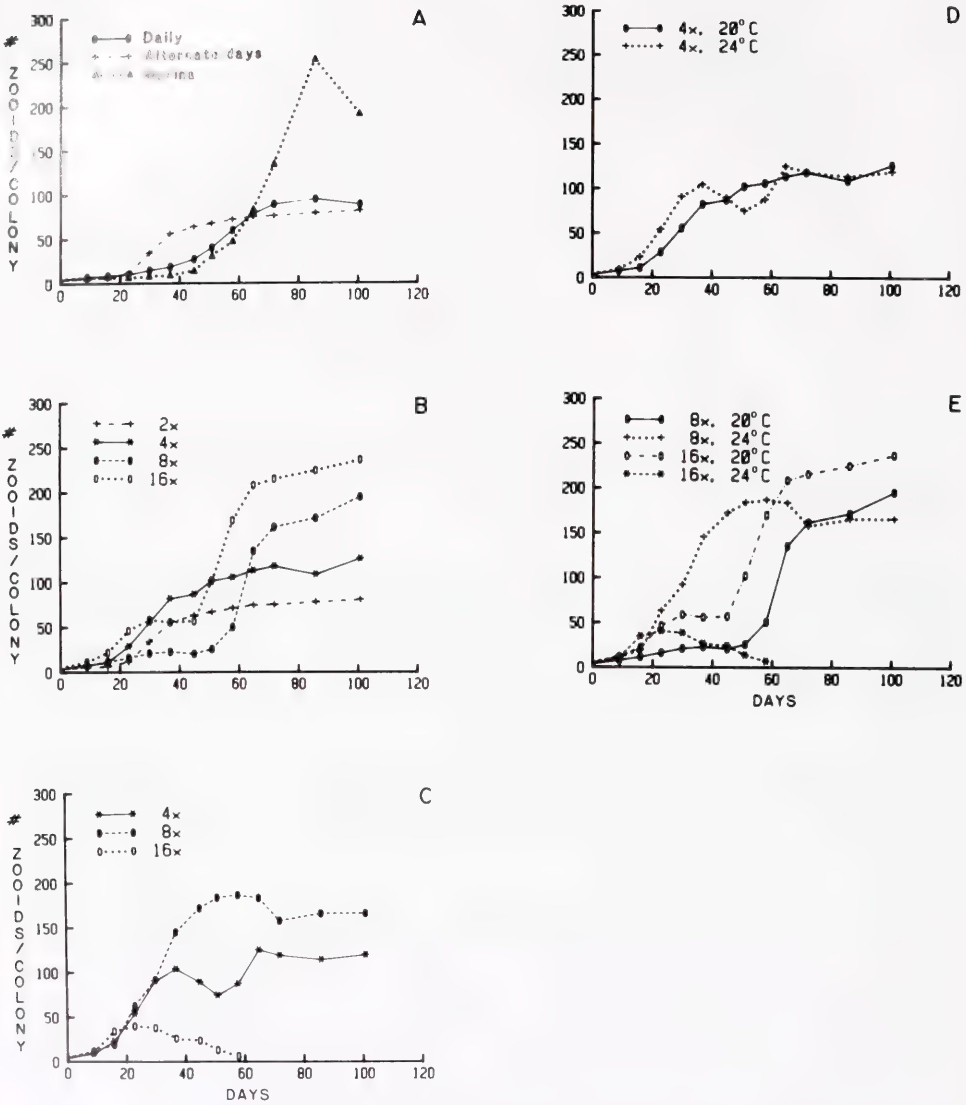

FIGURE 3. *Botryllus* growth curves for Phase II. The ordinate represents mean number of zooids per colony. Day 0 = 12/3/84. (Except as noted in A, laboratory treatments received alternate day seawater change.) A: Comparison of daily seawater change with alternate day seawater change for colonies fed 2× Liquifry at 20°C, and with the marina treatment. B: Colonies fed 2×, 4×, 8×, or 16× Liquifry at 20°C. C: Colonies fed 4×, 8×, or 16× Liquifry at 24°C. D: Colonies fed 4× Liquifry at 20°C and at 24°C. E: Colonies fed 8× or 16× Liquifry at 20°C and 24 °C.

colony sizes for the 4× Liquifry treatments at these two temperatures were fairly similar throughout Phase II (Fig. 3D), with differences observed only from day 16 to day 30 (Wilcoxon signed rank tests, $P < 0.05$). However, at the higher food levels, more prolonged differences were seen (Fig. 3E). Colonies fed 8× Liquifry grew much faster at 24°C than the 8×,20°C within the first two months of the experiment. Mean colony sizes were significantly different from days 23 to 58 (Wilcoxon signed rank tests, $P < 0.05$). On the other hand, colonies fed 16× Liquifry at 20°C grew much better than their 16×,24°C cohorts, which began a steady decline in growth after about three weeks in the observation period.

During the second month of this experiment the marina treatment's mean colony size (Fig. 3A) was generally smaller than those of most of the laboratory treatments. However, at about day 58 the marina curve began a sharp climb, similar to that seen during the second growth rise in the 8× and 16× Liquifry treatments at 20°C (Fig. 3B). Nevertheless, on day 86, at the peak of the marina curve, the mean size of its colonies was no different from that of colonies in any of the laboratory treatments (Kruskal-Wallis test: $H = 11.1$, d.f. = 7, $P > 0.10$). Because the two largest marina colonies died between days 86 and 101, this growth curve started to decline abruptly; survivorship at day 101 was about 70%.

Statistical analyses of Phase II growth data—maximum size. Two variables related to growth were examined by Friedman's tests and Wilcoxon-Wilcox multiple comparisons: (1) maximum size reached during the observation period; and (2) time elapsed to reach 50% maximum size in the experiment. Significant differences were observed among the treatments with respect to maximum size attained by individual colonies during the experiment. The Friedman's test result and the treatment rankings are shown in Table II. The order of treatments resulting from this statistical test is based on rank sums, and, due to variability among colonies, does not necessarily correspond to the numerical order determined by arithmetic means of the treatments. Some revised means were calculated which exclude anomalously large animals, and these means seem more appropriate for the ranks given (see footnote "c" in Table II). In practical terms, the results suggest that 16× Liquifry at 20°C is the best treatment for obtaining large colonies, whereas the 2×,20°C (daily) and the 16×,24°C are significantly worse.

Statistical analyses—growth rates. Growth rates were determined by recording days to reach 50% of each individual colony's maximum size in the experiment. Due to the poor growth of colonies in the 16×,24°C treatment, they were excluded from this analysis. Significant differences were observed among treatments for this parameter. The Friedman's test result and the treatment rankings are shown in Table III. Although the 16×,20°C colonies attained the largest maximum size during Phase II (Table II), this treatment was in the lowest range for days to reach 50% maximum size. On the

TABLE II

Results of Friedman's test and Wilcoxon-Wilcox multiple comparisons for maximum size (# zooids/colony) in Phase II

Friedman's test: Chi-square = 27.9 d.f. = 8 $P = 0.0005$

Treatment[a]	Mean ± S.E.	Group[b]
16×,20°C	262.4 ± 39.1	A
4×,24°C	147.8 ± 22.8	A B
8×,24°C	214.6 ± 76.3[c]	A B
Marina	299.6 ± 111.3[c]	A B C
4×,20°C	134.5 ± 28.2	A B C
8×,20°C	197.6 ± 73.9[c]	A B C
2×,20°C (alternate)	82.4 ± 19.5	A B C
2×,20°C (daily)	99.4 ± 28.8	B C
16×,24°C	47.6 ± 9.7	C

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details.

[b] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

[c] Mean ± S.E. when the few anomalously large animals were excluded (see Appendix) are as follows: 8×,24°C = 109.1 ± 19.6 (n = 12); Marina = 161.8 ± 93.5 (n = 12); 8×,20°C = 69.2 ± 11.4 (n = 10).

TABLE III

Results of Friedman's test and Wilcoxon-Wilcox multiple comparisons for days to reach 50% maximum size (# zooids/colony) in Phase II

Friedman's test: Chi-square = 72.4 d.f. = 7 $P = 0.0000$

Treatment[a]	Mean ± S.E.	Group[b]
4×,24°C	34.0 ± 5.0	A
8×,24°C	35.5 ± 2.3	A B
2×,20°C (alternate)	39.2 ± 2.6	A B C
4×,20°C	42.4 ± 4.3	A B C
2×,20°C (daily)	56.1 ± 1.9	B C D
16×,20°C	57.5 ± 2.5	C D
8×,20°C	67.7 ± 1.8	D
Marina	71.4 ± 8.7	D

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details. The 16×,24°C treatment was excluded from this analysis because its colonies grew poorly and this treatment was terminated early.

[b] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

other hand, the 4× and 8× treatments at 24°C yielded large colonies in a relatively short period of time (Tables II, III). When the daily *versus* alternate day seawater change treatments (2×,20°C) were compared in a separate Wilcoxon signed rank test, it was seen that colonies in the 2×,20°C (alternate) treatment reached 50% maximum size significantly faster than those in the 2×,20°C (daily) treatment ($Z = 3.2$, $P < 0.01$). Based on the rankings in Table III, the marina group grew more slowly than any of the laboratory treatment groups.

Since the colonies used in Phase II had a wide range of ages at the start of the experiment, additional Friedman's and multiple comparison tests were run separately for young and old colonies (age at day 0 = 18–32 days, n = 5 young colonies; age at day 0 = 46–58 days, n = 9 old colonies). With respect to maximum size attained by colonies during the experiment, the greatest ranking difference between young and old colonies occurred with the marina treatment, which was ranked as second for old colonies whereas it was ranked as seventh for young colonies. However, based on the distribution of the statistical ranges, we do not consider this difference to be significant. Statistical tests on data for days to reach 50% maximum size gave similar treatment rankings for young and old colonies.

Asexual generation time, which is reported to be influenced by temperature (Grosberg, 1982), was measured for three treatments in Phase II. Asexual generation time is the time interval which begins when a given zooid generation starts to function as filter-feeding adults and ends when this generation of zooids is completely resorbed and replaced by the new generation which arose by budding. The mean asexual generation times for the 4× Liquifry treatments were 5.5 days (standard error = 0.2 days, n = 8) at 20°C, and 4.1 days (standard error = 0.1 days, n = 14) at 24°C. For colonies grown in the marina, mean asexual generation time was 13.0 days (standard error = 1.0 days, n = 3).

Phase II: Colonies achieve sexual maturity under laboratory culture conditions faster than in the marina

Not only do certain culture conditions provide superior growth rates and sizes of *Botryllus* colonies, but a few of these treatments, notably 4× and 8× Liquifry at 24°C, allow the very early attainment of sexual maturity. In fact, all but one (16×,24°C) of the eight laboratory treatments in Phase II gave rise to a large proportion of sexually mature colonies during the observation period, with colonies grown at 24°C generally reaching sexual maturity earlier than those kept at 20°C. In contrast to the eventual attainment of sexual maturity by some marina colonies in Phase I (Fig. 2), the marina colonies did not produce eggs during Phase II.

The time course of appearance of eggs and/or embryos in colonies during Phase II is shown in Figure 4. In the 2×,20°C treatments, the initiation of sexual maturity in colonies receiving alternate day seawater changes preceded the production of eggs in daily seawater change colonies by two weeks; the percentage of sexually mature colonies in the 2×,20°C (daily) treatment remained lower than that of the 2×,20°C (alternate) treatment throughout Phase II (Fig. 4A). When comparing the four treatments at 20°C (Fig. 4B), one sees that colonies in the higher food levels (8× and 16× Liquifry) tended to lag behind colonies at the 2× (alternate) and 4× Liquifry levels with respect to onset of sexual maturity. The time courses for egg production in the 24°C treatments which bore sexually mature colonies (4× and 8× Liquifry) were fairly similar to each other, although initially the 8× food level did lag behind the 4× food level (Fig. 4C).

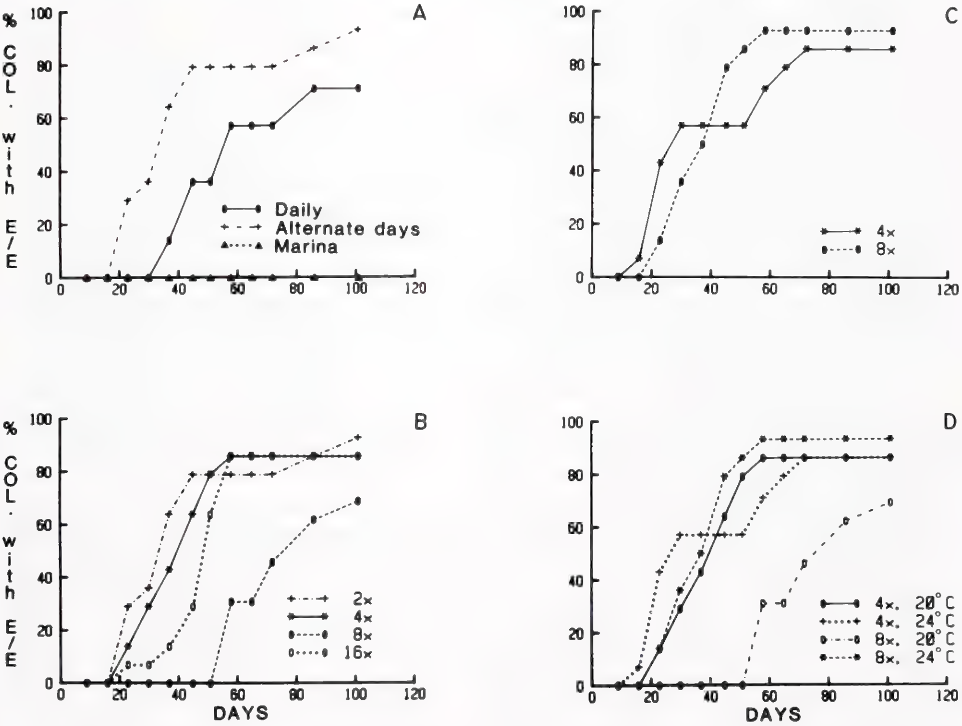

FIGURE 4. Sexual maturity of *Botryllus* colonies in Phase II. The ordinate represents cumulative percentage of colonies with eggs and/or embryos. Day 0 = 12/3/84. (Except as noted in A, laboratory treatments received alternate day seawater change.) A: Comparison of daily seawater change with alternate day seawater change for colonies fed 2× Liquifry at 20°C, and with the marina treatment. B: Colonies fed 2×, 4×, 8×, or 16× Liquifry at 20°C. C: Colonies fed 4× or 8× Liquifry at 24°C. (The 16×, 24°C treatment was terminated on day 58 due to poor colony condition; none of its colonies were sexually mature by this date.) D: Colonies fed 4× or 8× Liquifry at 20°C and 24°C.

TABLE IV

Results of Friedman's test and Wilcoxon-Wilcox multiple comparisons for days to reach sexual maturity in Phase II

Friedman's test: Chi-square = 57.4 d.f. = 7 $P = 0.0000$

Treatment[a]	Mean ± S.E.[b]	Group[c]
8×,24°C	37.5 ± 2.9	A
2×,20°C (alternate)	43.0 ± 6.3	A
4×,24°C	37.0 ± 5.8	A B
4×,20°C	43.4 ± 4.9	A B
16×,20°C	48.8 ± 3.0	A B C
2×,20°C (daily)	54.7 ± 5.9	B C D
8×,20°C	70.7 ± 4.9	C D
Marina	>101	D

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details. The 16×,24°C treatment was not included because it was terminated on day 58 and none of its colonies had become sexually mature by this date.

[b] For the Friedman's test, colonies which survived but did not become sexually mature by the end of the experiment were designated as occupying the highest rank. However, these means do not include such colonies.

[c] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

In looking at the time course of sexual maturity with respect to temperature at a particular food level, it was seen that colonies tended to become mature faster at 24°C than at 20°C, particularly at the 8× Liquifry level (Fig. 4D). The marina colonies failed to become sexually mature during the observation period (Fig. 4A).

Statistical analyses of attainment of sexual maturity. Significant differences among treatments with respect to time to reach sexual maturity were seen using Friedman's test and Wilcoxon-Wilcox multiple comparisons (Table IV). Colonies in the 2× (daily) and 8× Liquifry treatments at 20°C, along with the marina, took significantly longer to become sexually mature than colonies in the other laboratory treatments. In addition, we found significant differences among the treatments with regard to colony size at the onset of sexual maturity (Table V). The 16×,20°C treatment had the largest colonies at this time, and this treatment also ranked the highest for maximum size attained during Phase II (Table II). The smallest sexually mature colonies belonged to the two 2×,20°C treatments (Table V).

As with the growth data, the variables pertaining to sexual maturity were analyzed separately for young and old colonies. Treatment rankings for number of days required to reach sexual maturity in the experiment were nearly identical for young and old colonies. However, with the treatments pooled, young colonies took significantly longer to become sexually mature than old colonies (Wilcoxon rank sum test: $Z = 3.8$, $P < 0.01$). When considering size at sexual maturity, the treatment rankings for young and old colonies were very similar. When pooled, the mean sizes of young and old colonies at the onset of sexual maturity were not significantly different (Wilcoxon rank sum test: $Z = 0.9$, $P > 0.10$).

Phase II: Physical condition of colonies

Along with colony growth and attainment of sexual maturity, it was apparent that the physical condition of the colonies was an important character to consider. Figure 5 depicts the percentage of the initial number of colonies per treatment that were

TABLE V

Results of Kruskal-Wallis test and Wilcoxon rank sum tests for size (# zooids/colony) at sexual maturity in Phase II

Kruskal-Wallis test: H = 25.0 d.f. = 6 $P = 0.0003$

Treatment[a]	Mean ± S.E.	n[b]	Group[c]
16×,20°C	149.5 ± 21.5	11	A
8×,24°C	109.2 ± 25.2	13	A B
4×,24°C	89.8 ± 13.0	12	A B
8×,20°C	118.9 ± 42.2	9	A B
4×,20°C	78.3 ± 15.5	12	B
2×,20°C (daily)	44.7 ± 10.6	10	C
2×,20°C (alternate)	41.8 ± 10.1	13	C

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16× for the laboratory treatments. See Materials and Methods for details. There were no sexually mature colonies in the 16×,24°C or marina treatments, and thus these treatments were excluded.

[b] Decrease in n from an original total of 14 is due to mortality before attaining sexual maturity or failure to reach sexual maturity by the end of the experiment.

[c] Treatment means which do not share a group letter are significantly different ($P < 0.05$).

in satisfactory condition (see Materials and Methods) during Phase II. Changing the seawater every day *versus* on alternate days did not appear to affect colony condition (Fig. 5A). At 20°C, there was intermittent variation in colony condition among the Liquifry treatments (Fig. 5B). There was even greater variation in condition among the 24°C colonies (Fig. 5C). The condition of colonies in the marina (Fig. 5A) began to decline toward the end of the experiment. If the laboratory treatments are ranked according to the number of time points ($n = 13$) at which greater than 80% of the colonies were in satisfactory condition, one finds that for a given food level the 20°C treatment was better than the 24°C treatment, and that for a given temperature the lower food levels maintained colonies in better condition than did the higher food levels.

DISCUSSION

This paper describes the effects of different temperatures and diets on growth, condition, and sexual maturity in laboratory-cultured *Botryllus schlosseri* colonies from the Monterey Marina. Although other investigators have reported maintaining colonial tunicates on diets of various algae (Sabbadin, 1960; Milkman, 1967; Nakauchi *et al.*, 1979), our *Botryllus* colonies did not grow well with similar algae food sources (Fig. 1A). On the other hand, our Liquifry-fed colonies grew well, especially in Phase II (Fig. 3). This is an important result, as this food source eliminates maintenance and counting of algal cultures.

According to Milkman (1967), Woods Hole *Botryllus schlosseri* can grow at 18°C–28°C. After culturing young colonies of *B. schlosseri* from the Venetian lagoon at 18°C, 21°C, and 26.5°C, Sabbadin (1955) determined that 21°C gave very good results for asexual reproduction. In Japan, Nakauchi *et al.* (1979) found that 25°C allowed for the best growth of inland cultures of the colonial tunicate *Symplegma reptans,* but they had problems with bacterial contamination at that temperature, and thus 20°C became their temperature of choice. Since the normal annual water temperature range in Monterey Marina is about 12°C–17°C, we decided to set up initial comparisons of growth of our colonies at 15°C and 20°C, and found that the Liquifry-fed Monterey

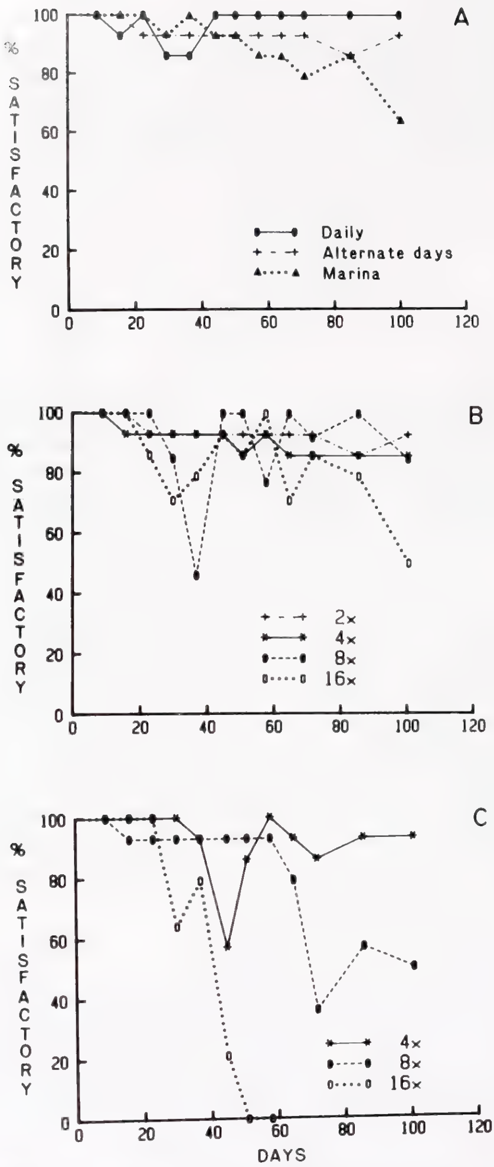

FIGURE 5. Percentage of *Botryllus* colonies in satisfactory condition during Phase II. Day 0 = 12/3/84. (Except as noted in A, laboratory treatments received alternate day seawater change.) A: Comparison of daily seawater change with alternate day seawater change for colonies fed 2× Liquifry at 20°C, and with the marina treatment. B: Colonies fed 2×, 4×, 8×, or 16× Liquifry at 20°C. C: Colonies fed 4×, 8×, or 16× Liquifry at 24°C.

Botryllus grew most rapidly at 20°C (Fig. 1B). Likewise, in Phase II the colonies grown at 24°C grew faster than those at 20°C (Figs. 3D, E; Table III), with the exception of the 16×,24°C treatment. This combination of high food and temperature levels allowed for the growth of many contaminating organisms in this treatment tank, which apparently contributed to the eventual demise of the *Botryllus* colonies.

Temperature is a major factor which influences sexual reproduction in marine invertebrates (Millar, 1971). Thus it was not surprising that the Liquifry-fed colonies at 20°C became sexually mature before those at 15°C in Phase I. The reduction in growth rate of colonies in the 20°C Liquifry treatments beginning around day 57 (Fig. 1B) coincides with the onset of egg production. It is possible that this is due to a shift in the partitioning of resources between asexual and sexual reproductive processes. Reports of this phenomenon occurring in colonies reared in their natural habitat have been made for *Botryllus* (Berrill, 1935; Mukai and Watanabe, 1976; Grosberg, 1982) and for *Trididemnum solidum* (Bak *et al.,* 1981). In Phase II, this decrease in growth rate at the onset of sexual maturity apparently also occurred in all but the 16×,20°C laboratory treatment (Figs. 3B, C; Table IV), which did not exhibit a reduced growth rate until about two weeks after sexual maturation began. In fact, the onset of sexual maturity in colonies in the 16×,20°C treatment occurs at the beginning of the second growth rise in that treatment. Thus it seems possible to provide laboratory-maintained *Botryllus* with sufficient food to develop into sexually mature colonies while continuing to increase in size via asexual reproduction.

Two interesting observations were made when comparing data from the daily *versus* alternate day seawater change treatments (2×,20°C) in Phase II. First, although their maximum sizes attained during Phase II were similar (Table II), colonies in the 2×,20°C (alternate) treatment reached 50% maximum size significantly more rapidly (39.2 ± 2.6 days, Table III) than those in the 2×,20°C (daily) treatment (56.1 ± 1.9 days, Table III) (Wilcoxon signed rank test: $P < 0.01$, see Results section). Second, although their colony sizes at onset of sexual maturity were similar (Table V), the colonies in the 2×,20°C (alternate) treatment became sexually mature earlier (43.0 ± 6.3 days, Table IV) than those in the 2×,20°C (daily) treatment (54.7 ± 5.9 days, Table IV; also see Fig. 4A) These observations have led us to speculate that growth factors and hormones which influence sexual maturation may have accumulated in the tank which received less frequent seawater exchange, thereby enhancing the rates of growth and attainment of sexual maturity of the colonies.

Upon completion of the Phase II culture experiments, the high degree of variability among Monterey *Botryllus* colonies with respect to growth rate and maximum size was evident (see Appendix; Table II). This phenomenon is of practical significance to investigators. The observed variation could be the result of different life history types, as described for *Botryllus schlosseri* in Woods Hole (Grosberg, 1982). In order to investigate the genetic contribution to such differences, one could set up matings between rapidly growing large colonies and slowly growing smaller colonies (three possible mating combinations) and study growth patterns of the offspring.

Our culture research provides ample evidence that laboratory conditions are more favorable than Monterey Marina for maintaining healthy sexually mature *Botryllus* colonies *year-round.* In addition to the results presented here, it was clear that laboratory-raised colonies were much cleaner than those grown in the field, where *Botryllus* must compete with other animals and plants for space. Clean colonies allow for more efficient experimental manipulations with respect to colony transfer and studies of fusion/rejection reactions. Furthermore, the marina environment is unpredictable; for example, the appearance within the last three years of persistent anomalously warm water currents originating off the coast of South America, called El Niño, has had significant biological consequences (Barber and Chavez, 1983), including causing changes in California coastal ecosystems (Dayton and Tegner, 1984). The fact that the marina environment is uncontrolled may have contributed to the sudden, sporadic death of our *Botryllus* colonies. The marina colonies had lower survivorships than the laboratory-grown colonies in both Phases I and II. When Phase II was terminated,

TABLE VI

Laboratory treatment conditions appropriate for some specified uses of Monterey Botryllus *colonies*

Purpose	Treatment conditions[a,b]
(1) Rapidly growing, large colonies for multiple fusion/rejection assays, or to provide material for protein and nucleic acid analysis.	(1) 4×,24°C 8×,24°C 4×,20°C
(2) Colonies with early onset of sexual maturity to develop inbred strains.	(2) 8×,24°C 2×,20°C 4×,24°C
(3) Colonies of sufficient size with early onset of sexual maturity to produce many offspring from defined matings.	(3) 8×,24°C 4×,24°C 16×,20°C
(4) Rapidly growing colonies with early onset of sexual maturity to produce several generations of defined stocks.	(4) 8×,24°C 4×,24°C 2×,20°C
(5) To hold colonies in slow growth until future use.	(5) 2×,15°C

[a] Liquifry concentrations are designated as 2×, 4×, 8×, 16×, for the laboratory treatments. See Materials and Methods for details.

[b] The top three treatments are listed in order of preference for each purpose. However, one should remember that colony condition remains good for longer periods of time at the lower food and temperature levels.

the remaining laboratory treatment colonies were transferred to the 4× Liquifry, 20°C condition. Six weeks later, survivorship of these colonies was 85%, whereas only approximately 30% of the marina colonies were still alive. Another advantage of laboratory-reared colonies is that since sexually mature colonies produce new eggs during each asexual cycle, we can obtain two or three times as many sets of progeny from laboratory colonies as compared with matings between marina-maintained colonies in a given time interval, due to the shorter asexual generation times at 20°C and 24°C. This is a significant advantage for the production of genetically defined inbred strains.

We now know how to construct laboratory conditions to obtain *Botryllus* colonies with various life-cycle characteristics to satisfy different experimental needs, as outlined in Table VI. Because it is likely that the high degree of variation among colonies with respect to growth is genetically determined, we will interbreed the healthy rapidly growing colonies as soon as they become sexually mature in order to accomplish our goal of establishing long-term defined genetic stocks in the laboratory. These stocks will be invaluable tools for *Botryllus* research in areas such as immunogenetics, developmental biology, and ecology.

ACKNOWLEDGMENTS

We appreciate the technical assistance of Katherine Ishizuka, Anita Nynas-McCoy, and Susan Ensminger. We thank J. Danska, S. Gaines, and J. Watanabe for their comments on the manuscript. Support for this investigation was provided by NIH grant R01-GM25902-05, USPHS fellowship T32-CA09302-06 and California Division-American Cancer Society Junior Fellowship #J-7-84 (H.C.B.), Department of Energy Contract EV10108, and by a fellowship from the Cancer Research Institute, New York (J.A.H.).

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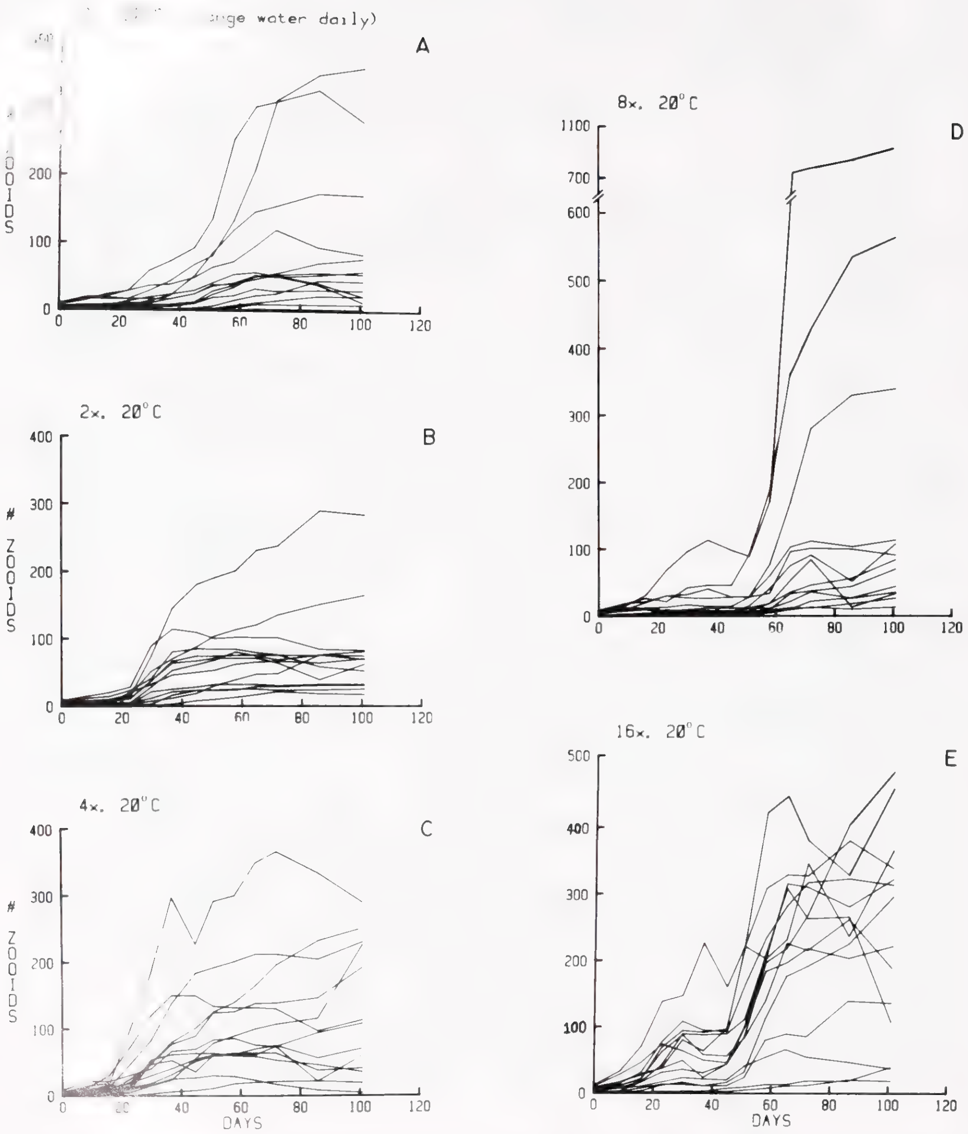
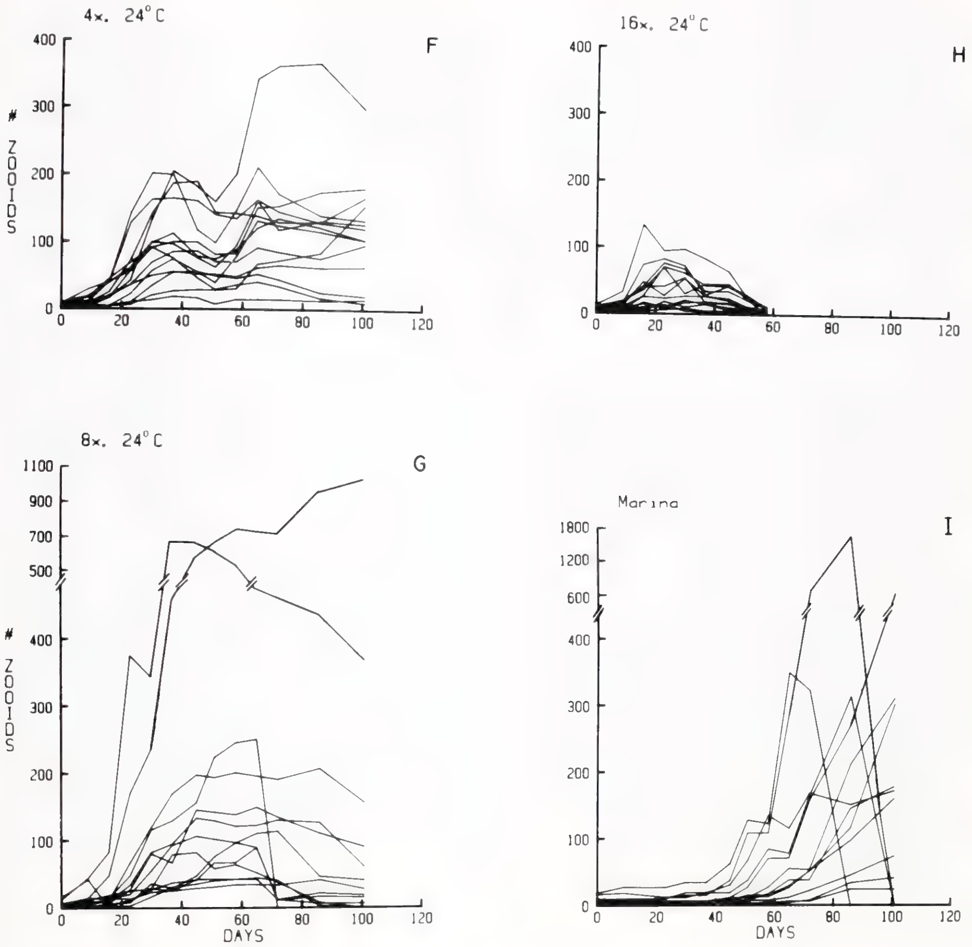
APPENDIX



Growth curves of individual *Botryllus* colonies for the nine treatments in Phase II. Day 0 = 12/3/84. Liquifry concentration is designated as 2×, 4×, 8×, or 16×. (Except as noted in A, all laboratory treatments received alternate day seawater change.)



APPENDIX (*Continued*)

Reference: *Biol. Bull.* **170**: 110–121. (February, 1986)

SYMBIOTIC ASSIMILATION OF CO_2 IN TWO HYDROTHERMAL VENT ANIMALS, THE MUSSEL *BATHYMODIOLUS THERMOPHILUS* AND THE TUBE WORM *RIFTIA PACHYPTILA*[1]

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ABSTRACT

Shipboard studies showed major differences in the incorporation of CO_2 by the specific prokaryotic symbionts of two deep-sea vent invertebrates. The rate of CO_2 fixation was optimal at approximately 22°C in fresh trophosome material from the pogonophoran tube worm *Riftia pachyptila.* Sulfide, but not thiosulfate, served as the electron donor. Thirty-five percent of the aerobic rate remained in deoxygenated samples presumably due to traces of hemoglobin-bound oxygen. Gill preparations from the mytilid mussel *Bathymodiolus thermophilus,* however, assimilated CO_2 with a maximum rate at 12° to 15°C. Thiosulfate, but not sulfide, served as the electron donor. The activity was completely inhibited in deoxygenated samples. These metabolic dissimilarities between the symbionts of the two hosts extend to DNA base ratios and cell sizes. Partial fractionation of the mussel gill preparation yielded a prokaryotic or "bacterial" fraction that showed a CO_2 assimilation rate three-fold higher than that of the crude homogenate. This activity was not affected by the *in situ* hydrostatic pressure of 250 atm (100% barotolerance). The mussel gill symbiont represents the first sulfur-oxidizing vent prokaryote with psychrophilic growth characteristics. The natural distribution of *R. pachyptila* and *B. thermophilus* within the physico-chemical regime of the immediate vent vicinity corresponds well with the metabolic differences of their symbionts.

INTRODUCTION

The extensive animal populations found at hydrothermal vents along the East Pacific Rise fracture zone were proposed to be supported by chemosynthetically produced organic carbon (Lonsdale, 1977; Corliss *et al.,* 1979; Jannasch and Wirsen, 1979). Indeed, during the first biological studies, the mixing zone of the highly reduced hydrothermal fluid with the ambient, oxygen-containing seawater was found to support large numbers of chemoautotrophic bacteria (Jannasch and Wirsen, 1979, Karl *et al.* 1980).

From their unusual abundance it was apparent that certain vent animals derived their food supply by means other than filtration of suspended bacterial cells. The symbiotic nature of their nutrition was soon proved by morphological and/or enzym-

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We dedicate this paper to Professor Norbert Pfennig, University of Konstanz, in honor of his 60th birthday.

Received 25 September 1985; accepted 25 November 1985.

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ological studies (Cavanaugh *et al.,* 1981; Felbeck, 1981; Felbeck *et al.,* 1981; Cavanaugh 1983). While the giant white clam, *Calyptogena magnifica* (Boss and Turner, 1980), and the mytilid mussel, *Bathymodiolus thermophilus* (Kenk and Wilson, 1985), appear to harbor prokaryotic cells within their gill tissue, these cells are concentrated as a separate tissue, the trophosome, within the coelomic cavity of the mouth- and gutless pogonophoran tube worm *Riftia pachyptila* (Jones, 1981).

These three invertebrates are the most conspicuous of the vent animals, but their relative abundances differ from vent to vent. *Calyptogena* and *Riftia* dominated at the "21°N" site (East Pacific Rise) with the mussel being absent. By contrast, clusters of tube worms and mussels composed most of the biomass in other vent sites (Grassle, 1985). Of these locations, the highest standing animal crop was reported at the site known as "Rose Garden" in the Galapagos vent system (Hessler and Smithey, 1983). This site was revisited in March 1985.

During the latter expedition we studied the assimilation of ^{14}C-labeled bicarbonate by homogenized *Riftia* trophosome and *Bathymodiolus* gill tissue focussing on the effects of temperature, oxygen, pressure, and the type of sulfur compound used as the energy source.

MATERIALS AND METHODS

Collection and storage of specimens

During the March 1985 Galapagos expedition, live animals were collected at a depth of 2550 m at the "Rose Garden" vent site (0°84.25′N, 86°13.48′W) on 14 out of 18 DSRV ALVIN dives. Upon collection, the animals were placed in a closed insulated container designed to keep the contents at deep sea temperature (*ca.* 3°C) during the submersible's ascent. A minimum of 2 h elapsed between collection of animals and their availability on the surface. Immediately upon return to the surface, the animals were removed, placed on ice in a 4°C refrigerator, and dissected for the experiments within one hour. Only live animals, as evidenced by the tight closure of the shells (*Bathymodiolus*), or by the characteristic withdrawal into the tube (*Riftia*), were used.

Medium

For all experiments, an artificial seawater medium (ASW), as described by Ruby and Jannasch (1982), was used with the concentration of $NaHCO_3$ raised to 2.5 m*M* and the Tris omitted. A phosphate buffer (3.0 mmolar) was sufficient to keep the pH constant at 7.5 at all experimental conditions.

Homogenate preparation

Mussel gills were excised from the animal taking care not to include fragments from other tissues. Prior to homogenization, the gills were rinsed three times with 40 ml sterile seawater. Trophosome tissue, which was too loose in texture to be similarly washed, was removed aseptically with minimal amounts of worm tissues present.

These tissue samples, approximately 5 ml in volume, were mixed with equal volumes of 4°C sterile ASW and gently homogenized in an ice-cooled ground glass homogenizer. The protein concentration of the mussel gill preparation ranged from 18 to 27 $mg \cdot ml^{-1}$, and that of the worm trophosome between 60 to 97 mg $protein \cdot ml^{-1}$. The homogenate was kept on ice until its dilution into the reaction medium, usually for not more than 10 min.

Experiments

The concentrated homogenates were diluted 100-fold in 4°C ASW and distributed in 15 to 20 ml aliquots to 50 ml rubber-stoppered flasks. After preincubation at selected temperatures for 10 to 20 min, $NaH^{14}CO_3$ (50 $\mu Ci \cdot ml^{-1}$) was added in various amounts to yield final specific activities of 0.05 to 0.10 $mCi \cdot mmol^{-1}$. At appropriate intervals, 2 to 3 ml samples of the suspension were filtered through 25 mm GF/F filters (Whatman), and rinsed with 3 volumes of 4°C sterile seawater. The filters were then fumed for 20 min over concentrated HCl and dried in air. The radioactivity incorporated into the cells was measured with a Beckman liquid scintillation counter (Model LS 100C).

To achieve O_2-free conditions, ASW, prior to $NaHCO_3$ addition, was bubbled with O_2-free N_2 gas for one hour. The homogenate and $NaHCO_3$ (labeled as well as unlabeled) were then added, and 15 ml aliquots were distributed to 17 ml Hungate test tubes (Bellco Glass, Inc.). All the above steps were carried out on ice under a nitrogen gas flow. A 20 ml portion was transferred to a flask and aerated to serve as an aerobic control.

To measure the effect of pressure, 3.5 ml serum stoppered glass tubes were completely filled with well aerated, pre-mixed reaction medium. The tubes were then either left at 1 atm or placed into stainless steel pressure cylinders which were immediately sealed and pressurized to 250 atm. The time between isotope addition and attaining full pressure was approximately 5 min. At each point, samples were treated as described above.

Fractionation of mussel gill homogenate

Five fractions of mussel gill homogenate were prepared as follows: The crude homogenate was centrifuged for 5 min at low speed (3000 × *g*) in a table-top centrifuge. The pellet was resuspended in ASW medium and recentrifuged as before. The two supernatants were combined, and centrifuged for 6 min in an Eppendorf microfuge (Model 5414) at 15,000 × *g*. The resulting five fractions were: the crude homogenate, the low and high speed supernatants, and the pellets of the low and high speed centrifugations. The last of these was designated the "purified" bacterial fraction.

Protein determination

Aliquots of tissue homogenates were supplemented with trichloroacetic acid (10% w/v final concentration) and protein was precipitated by heating (90°C, 20 min). After pelleting at room temperature (15,000 × *g*, 10 min) protein was dissolved in 0.1 *M* NaOH (50°C, 30 min) with several gentle mixings. It was then measured by the Coomassie Brilliant Blue dye binding technique of Bradford (1976) using a microassay and commercially available dye reagent as previously described (Nelson *et al.*, 1982).

Sulfide preparation and determination

A 200 m*M* stock solution of Na_2S was prepared after the method of Brock *et al.* (1971) and autoclaved. A volume was neutralized with HCl just before use and added to experimental flasks at 100 to 1200 μM final concentration. At the conclusion of the cruise, the stock solution still contained 93% of its initial sulfide concentration as determined by the method of Cline (1969). The sulfide concentrations stated for our experiments are only valid for the start of the experiment as sulfide at neutral pH in aerobic seawater has a half-life of approximately 1 h (Almgren and Hagström, 1974).

DNA

DNA was extracted from homogenized gill tissue (2.5 g wet wt) and from the "purified" bacterial fraction (0.75 g wet wt). It was deproteinized, purified by repeated washings after binding to hydroxylapatite, and dialyzed. These procedures, as well as methods for estimating base composition, genome size, and relative proportion of more rapidly renaturing DNA, were as described previously (Nelson *et al.*, 1984).

RESULTS

Electron donor

Figure 1 represents the data of experiments testing the capacity of two sulfur compounds, Na_2S and $Na_2S_2O_3$, to support CO_2 incorporation in mussel gill and in worm trophosome homogenates. It appears that the *Bathymodiolus* gill tissue can utilize thiosulfate but not sulfide while the pattern is reversed in the *Riftia* trophosome tissue. In both cases poisoned samples (1.5% glutaraldehyde) and homogenized host tissues (mussel mantle or worm vestimentum) showed CO_2 incorporation which did not increase beyond the "zero time" level. For *Bathymodiolus* (data not shown) these values were less than 0.5 nmol CO_2 mg^{-1} protein in assay mixtures supplemented with 400 μM $Na_2S_2O_3$.

While the enhancing effects of thiosulfate and sulfide were reproducible, a certain degree of variability was observed in both cases in the activity of the unsupplemented preparations. This correlated with the length of the preincubation period which preceded the introduction of the radioactive substrate. The results in Figure 1 were obtained after approximately 15 min of preincubation. When this period was increased to 75 min, no activity was detected in the mussel preparations without thiosulfate.

In addition to electron donor specificity, other differences between the two active preparations were in the reaction's duration and linearity. Whereas the activity of mussel gill tissue was linear for 2 to 3 h or more, that of the *Riftia* trophosome prep-

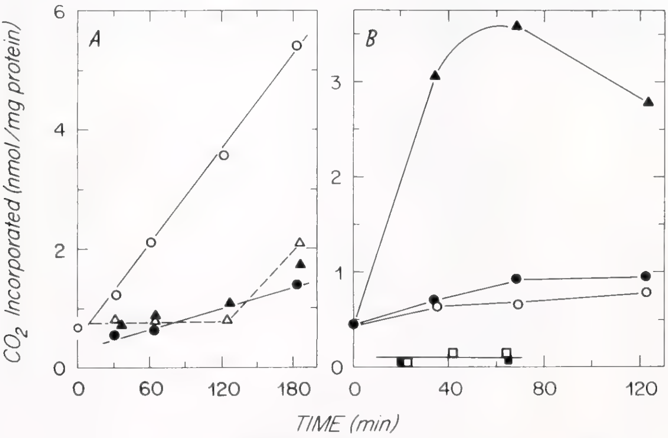

FIGURE 1. Incorporation of CO_2 in *Bathymodiolus* gill (A) and *Riftia* trophosome (B) homogenates in the presence of two electron donors. A) (○) 400 μM $Na_2S_2O_3$; (▲) 100 μM Na_2S; (△) 500 μM Na_2S; (●) no additions (tests were done at 6°C and the diluted homogenate contained 240 μg protein ml^{-1}). B) (○) 500 μM $Na_2S_2O_3$; (▲) 600 μM Na_2S; (■) 600 μM Na_2S plus 1.5% glutaraldehyde; (●) no additions (tests were done at 12°C and the diluted homogenate contained 600 μg protein ml^{-1}); (□) diluted vestimentum homogenate (850 μg protein ml^{-1}) plus 600 μM Na_2S.

aration was linear for less than 1 h. The incorporated $^{14}CO_2$ actually decreased after that time period (Fig. 1B). This phenomenon, which may indicate cell lysis or leakage of labeled solutes, could not be prevented or postponed by the addition of fresh sulfide after 4[illegible] min, the approximate time at which no more sulfide could be detected in the medium.

Various concentrations of the two electron donors were tested, and the results are presented in Figure 2. The mussel gill homogenate was equally active with all thiosulfate concentrations tested (100 *μM* or higher), although it was not determined whether higher concentrations may support the activity for longer periods of time. The optimal sulfide concentration for the *Riftia* trophosome was approximately 600 *μM*. At 1.2 m*M* sulfide the rate of CO_2 incorporation was about 40% of the optimal, but was relatively short in duration (20 min).

Temperature

The data of Figure 3 show a distinct difference in the temperature range favored by the two crude preparations. The "purified" fraction of mussel gill preparation behaved very similarly, if not identically, to the crude homogenate. Optimal temperature for CO_2 incorporation was 15°C or lower (four separate experiments) for the mussel and approximately 22°C for the worm. At 0°C the worm preparation exhibited about 5% of maximal activity while the mussel preparation was significantly more active. In four temperature profiles for *Bathymodiolus* homogenate, the CO_2 incorporation at 0°C averaged 42% (±17%) of the maximum. At the other end of the temperature range tested, the trophosome preparation was still active at 28° to 30°C, whereas the mussel preparation became inactive at approximately 20°C.

Oxygen

In view of the different redox potentials conferred by the two sulfur compounds serving as electron donors, the rate of CO_2 incorporation was tested for its response to the availability of O_2, the oxidizing agent commonly used by chemoautotrophic bacteria. As demonstrated in Figure 4A, the crude mussel gill preparation showed no

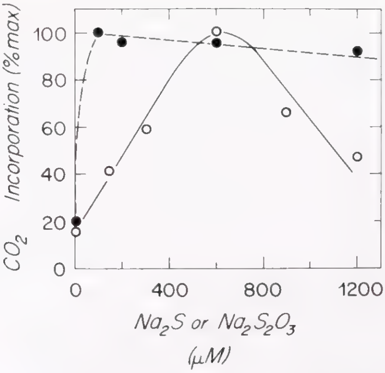

FIGURE 2. Incorporation of CO_2 in *Bathymodiolus* gill (●) and *Riftia* trophosome (○) homogenates at various concentrations of thiosulfate and sulfide respectively (tests were done at 6° and 12°C and the diluted homogenates contained 240 and 720 μg protein ml^{-1}, respectively). Maximum CO_2 incorporation rates in these experiments were 1.7 and 2.0 nmol mg^{-1} protein h^{-1}, respectively.

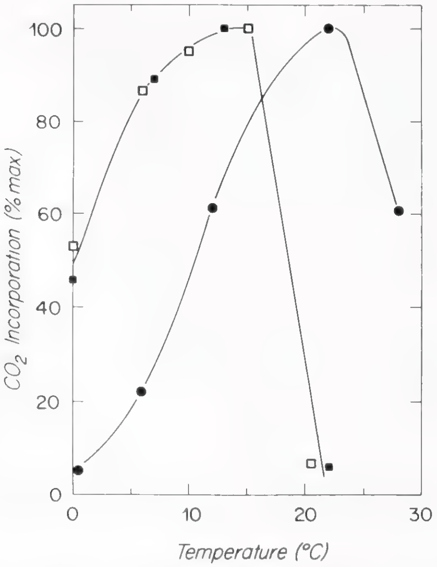

FIGURE 3. Incorporation of CO_2 in *Bathymodiolus* gill and *Riftia* trophosome homogenates at various temperatures; (□) mussel gill homogenate incubated with 200 μM $Na_2S_2O_3$; (■) "purified fraction" of mussel gill homogenate incubated with 400 μM $Na_2S_2O_3$; (●) trophosome homogenate with 600 μM Na_2S. Maximal rates in these experiments were 6.0, 1.0, and 3.3 nmol CO_2 mg^{-1} protein h^{-1}, respectively.

activity under anaerobic conditions. The activity of the *Riftia* preparation also was reduced, but only by approximately two-thirds (Fig. 4B). Here, differences in incorporation between duplicate "anaerobic" preparations (at or before arrow) may reflect differences in the amount or availability of oxygen bound to traces of *Riftia* blood. The short-term stimulating effect of aeration appears to be significant in restoring CO_2 incorporation to its initial rate for a period of approximately 20 min.

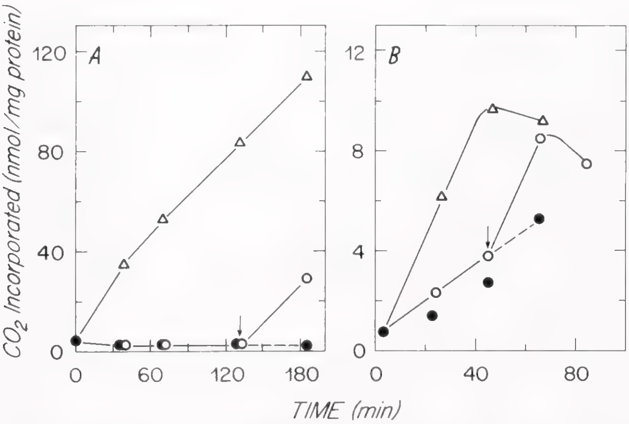

FIGURE 4. Incorporation of CO_2 in *Bathymodiolus* gill "purified" preparation (A, 320 μg protein ml^{-1}, incubated at 8°C with 400 μM $Na_2S_2O_3$) and *Riftia* trophosome homogenate, (B, 970 μg protein ml^{-1} incubated at 14°C with 600 μM Na_2S) under (●) N_2, (○) N_2, air introduced at arrow, and (△) air.

"Purified" Bathymodiolus *gill homogenate*

Since the *Riftia* preparation proved to be very unstable, we concentrated our purification efforts on the mussel gill homogenates. The activities measured with the "purified" preparation (see Materials and Methods) were very similar to those of the crude homogenate with respect to their response to temperature (Fig. 3) and to the utilization of thiosulfate as the electron donor (Fig. 4). The specific activity of the "purified" fraction was up to 3-fold higher than those of the crude homogenates (30.2 *vs.* 9.4 nmol CO_2 mg^{-1} protein h^{-1}).

Appropriate dilutions of all five fractions (see Materials and Methods) were assayed for CO_2 incorporation, measured for protein, and also stained (acridine orange) for examination using epifluorescence microscopy (Hobbie *et al.,* 1977). This examination revealed highly stained spheres or short rods of uniform appearance having a diameter of 0.5 to 0.75 μm. It was calculated that there were approximately 5×10^{10} of these cells per cm^3 of intact gill tissue. When CO_2 incorporation of each of the five fractions was normalized to the number of these cells present, values within the narrow range of $0.7\text{–}1.8 \times 10^{-8}$ nmol CO_2 $cell^{-1}h^{-1}$ were obtained. On the other hand, CO_2 incorporation expressed per unit protein varied by over 200 fold (0.1–23 nmol CO_2 mg^{-1} protein h^{-1}) for the same five fractions.

Pressure

The CO_2 incorporation by the "purified" fraction of the mussel gill homogenate was measured at the *in situ* pressure (250 atm) of the animal's habitat and compared to a 1 atm control. The data of Figure 5 show that, at least over the 1 h period, the applied pressure appears to have no significant effect on the measured activity.

DNA

The host DNA in the mussel gill has a base ratio of approximately 28 to 33 mol % guanine + cytosine (% G + C). The symbiont DNA has approximately 34 to 38% G + C and a genome size within 20% of that of *E. coli.* These assignments are based on the observation that the higher G + C content DNA became more abundant in the "purified" fraction with a parallel increase in the fraction of DNA possessing faster

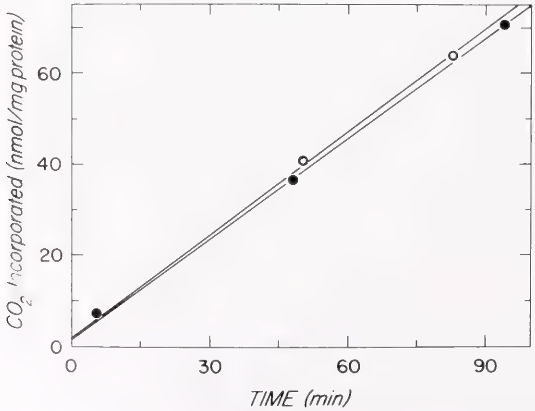

FIGURE 5. Incorporation of CO_2 in the "purified" fraction of *Bathymodiolus* gill homogenate (380 μg protein ml^{-1}) at 9°C with 400 μM $Na_2S_2O_3$, (●) at 1 atm and (○) at 250 atm.

TABLE I

Characterization of DNA of hosts and symbionts by the method of thermal denaturation and renaturation

	Host	Symbiont	
Name	Base ratio (mol % G + C)	Base ratio (mol % G + C)	Genome size[a] ($\times 10^9$ dalton)
Riftia pachyptila[b]	34–44	57–59	2.1–2.2
Bathymodiolus thermophilus[c]	28–33	34–38	1.9–2.0

[a] *Escherichia coli*, strain 620 K-12F (genome = 2.4×10^9 dalton), used as standard.

[b] From Nelson *et al.*, 1984.

[c] Based on DNA extracted from (1) crude homogenate, *B. thermophilus*, (2) "purified" friction, *B. thermophilus*, and (3) *E. coli* standard. Genome size calculated assuming that 90% of DNA of "purified" fraction was from symbiont.

(non-eukaryotic) renaturation kinetics. Both of these changes paralleled the higher CO_2 fixing capacity of the "purified" fraction. These data are presented (Table I) along with corresponding data for *Riftia* and its symbiont. Of the DNA obtained from homogenized trophosome tissue, approximately 90% was that of the symbiont (Nelson *et al.*, 1984). By contrast, symbiont DNA comprised only about one-third of the DNA obtained from the crude homogenate of *Bathymodiolus*.

DISCUSSION

In view of the absence of symbiont isolates, the data reported in this paper are hoped to help in elucidating the types and the mechanisms of the symbiotic systems in *Bathymodiolus thermophilus* and *Riftia pachyptila*. In both cases the CO_2-fixing tissue (gill or trophosome) contained a significant fraction of prokaryotic DNA as indicated by their genome sizes (Table I). The latter were well within the range for those of free living prokaryotes (1 to 3×10^9 dalton) and considerably greater than those of obligate symbionts (Soldo *et al.*, 1983). While sufficiently large genome sizes do not guarantee that either symbiont may eventually be grown free of its host, they imply that the present lack of success cannot be ascribed to a major loss of genetic information.

The two symbionts also differed in their DNA base compositions, the *Riftia* symbiont falling within the range characteristic of the genus *Thiobacillus* and the *Bathymodiolus* symbiont within that characteristic of *Thiomicrospira* (Kuenen and Tuovinen, 1981). Furthermore, the prokaryotic DNA comprised only about one third of the total *Bathymodiolus* gill DNA, while approximately 90% of the *Riftia* trophosome DNA was prokaryotic (Table I). Similarly, in a study of the 5S ribosomal RNA sequence in *Riftia* trophosome tissue, Stahl *et al.* (1984) found that approximately 75% of the total RNA was prokaryotic. These different proportions of eukaryotic *versus* prokaryotic material within the two hosts appears to be logical considering that the *Riftia* trophosome is believed to be specialized for harboring the symbionts whereas the mussel gill carries out other functions as well.

Chemoautotrophy, typical of sulfur oxidizing bacteria, was demonstrated by the effects of appropriate electron donors and oxygen on the incorporation of CO_2 into organic cell carbon. No CO_2 incorporation was found in the controls, *i.e.*, mantle tissue of *Bathymodiolus* and vestimentum tissue of *Riftia*. In the homogenized, symbiont-containing tissues the maximal CO_2 fixation rates were 44 and 13 nmol C mg^{-1} protein h^{-1} for *Bathymodiolus* and *Riftia*, respectively. Our assertion that these rates measure principally autotrophic fixation is strengthened by the observation that they are of the same order of magnitude as the maximum ribulose-1,5-sebisphosphate carboxylase activities measured in these tissues by others (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981) when based on the reasonable assumption that 1.0 g of tissue (wet wt) contains approximately 100 mg of protein. We presume that the variability in CO_2 incorporation rates measured in this study (see Figs. 2, 3) reflects different percentages of symbiont cells which were broken during tissue homogenization, contributing measurable amounts of protein but no CO_2 fixation.

The constancy of CO_2 incorporation per cell in the five preparations of *Bathymodiolus* gill homogenate can be taken as evidence for autotrophy in the mussel symbiont. Using a conversion factor of 1.2×10^{-13} gC μm^{-3} (Hagström, 1984), their average diameter of 0.6 μm translates into an approximate carbon content of 1.1×10^{-6} nmol per cell. This amount represents 60 to 140 times the hourly fixation rate per cell. These values imply relatively long doubling times, especially when compared to those of less than one hour as measured in free-living sulfur bacteria isolated from hydrothermal vents (Jannasch *et al.*, 1985).

The *Riftia* symbiont appears to be a short rod or sphere with diameters of approximately 3–5 μm (Cavanaugh *et al.*, 1981). The mussel gill symbiont, as noted above, is considerably smaller. These morphological differences between the two symbionts are paralleled by differences in their DNA base ratios, their primary use of different sulfur compounds as electron donors, their dissimilar temperature optima, and their response toward the presence of free oxygen.

Some of these differences may be reflected by the locations at which the two host animals are most commonly observed in the immediate vicinity of the vents. *Riftia* populations occur primarily near orifices of "warm" vents (Hessler and Smithey, 1983; Grassle, 1985; pers. obs.) where temperatures are in the range of 10 to 20°C and sulfide is prevalent (Edmond *et al.*, 1982; Jannasch and Mottl, 1985). Although mussels are often found intermingled with populations of tube worms, their bulk occurrence in dense beds immediately outside (with respect to vent orifices) of worm populations is in accordance with the psychrophilic characteristics of the *Bathymodiolus* symbiont.

It is of special interest that the *Bathymodiolus* symbiont appears to be the first sulfur-oxidizing prokaryote with truly psychrophilic growth characteristics. All free-living sulfur-oxidizing bacteria isolated so far from vent environments are mesophilic (Ruby and Jannasch, 1982; Jannasch *et al.*, 1985). The species designation of the mussel *B. thermophilus* (Kenk and Wilson, 1985) was based on its occurrence near warm vents and was published before the psychrophilic characteristics of their symbionts were found.

Additional evidence that the presence of sulfide within vent plumes is of more importance for the metabolism of *Riftia* than for that of *Bathymodiolus* is provided by enzymological data. Using a recently described assay (Powell and Somero, 1985) it was determined that sulfide oxidase activity in worm trophosome was 20 to 40 times higher than in the mussel gill tissue (Powell and Somero, in prep.). This is in accordance with the observation that no sulfide-binding protein, such as the one characteristic of *Riftia*, was found in *Bathymodiolus* preparations (J. J. Childress, pers. comm.).

The source of the thiosulfate used by the *Bathymodiolus* symbiont *in situ* is a point which is yet to be clarified. To our knowledge, no determinations of this anion in vent water were reported. The spontaneous oxidation of sulfide in seawater is known to proceed with a half-life of approximately one hour, with thiosulfate being a principal product (Almgren and Hagström, 1974). Thiosulfate can also be thermally produced from elemental sulfur (Belkin *et al.*, 1985). The fact that sulfide-stimulated CO_2 incorporation appears to commence eventually in the mussel symbiont (Fig. 1A, dashed line) suggests a thiosulfate-stimulation in disguise.

The *Bathymodiolus* symbiont appears to require free oxygen and incorporates CO_2 even at an atmospheric partial pressure of oxygen. The lack of a sustained aerobic CO_2 incorporation by the *Riftia* symbiont (Fig. 1B and unpub. obs.) cannot be attributed to an insufficient supply of sulfide because its replenishment did not prolong the period of linear incorporation. A similarly short-lived sulfide oxidation by trophosome preparations was observed by Fisher and Childress (1984).

A satisfactory explanation of the apparent anaerobic CO_2 incorporation by the *Riftia* symbiont (Fig. 4B) still requires a more detailed study. At this point, the data appear to indicate that free oxygen may be entirely replaced by hemoglobin-bound oxygen as proposed earlier (Arp and Childress, 1983). On the other hand, microaerophilism, a requirement for free oxygen at exceedingly low levels, is a common characteristic among the chemoautotrophic sulfur bacteria (Ruby and Jannasch, 1982; Jorgensen and Revsbech, 1983; Nelson and Jannasch, 1983).

Our recent observations at the Galapagos Rift vent site (May 1985) indicated profound environmental changes during the years elapsed since previous visits (January and December 1979). At the "Rose Garden" vent site, the extended "forests" of *Riftia* had all but disappeared and were replaced by extended mussel beds. *Riftia* occurred almost exclusively in two clumps each approximately 1 m in diameter. The maximum temperature in these limited worm populations was measured as 18°C (J. J. Childress, pers. comm.) and the concentration of H_2S was up to 300 μM (K. S. Johnson, pers. comm.). In contrast, the maximum temperature measured below mussels was 12°C and frequently dropped to ambient temperature of 2°C directly above the mussel beds (R. Lutz, pers. comm.). The sulfide concentration in the vicinity of mussels ranged from 0 to 100 μM (K. S. Johnson, pers. comm.). It appears that the remaining areas of strongest vent flow favored the maintenance of *Riftia* populations while the increasing dominance of *Bathymodiolus* may be a result of general decrease of the local hydrothermal vent activity. Our data indicate that these population changes reflect the specific metabolic properties of the two different chemoautotrophic symbionts.

Growing the symbionts in pure culture will still be necessary for their ultimate characterization. So far, isolation attempts from *Riftia* trophosome material resulted in organisms that did not resemble the symbionts in their diagnostic characteristics (Jannasch and Nelson, 1984). Studies, such as the present one, of the conditions under which prokaryotic preparations do sustain high rates of CO_2 incorporation, will aid further isolation attempts. Micro-oxic conditions and perhaps the involvement of oxygen-saturated *Riftia* blood possessing a sulfide-binding capacity (Arp and Childress, 1983) might have to be used. Since *Riftia* specimens are sensitive to a loss of pressure (Childress *et al.,* 1984), the possibility exists that its symbiont exhibits some barophilic characteristics. On the other hand, a broad pressure tolerance of prokaryotes, in general, as found so far in all sulfur-oxidizing bacteria isolated from the vents (Jannasch, 1984), might be offset by a pressure sensitivity of various host-symbiont interactions.

More optimism is justified in isolation experiments on the *Bathymodiolus* symbiont. Its sustained aerobic CO_2 incorporation at atmospheric pressure and its use of thiosulfate as an electron source render chances of its cultivation not more remote than that of similar symbionts now also found in shallow water clams (Cavanaugh, 1983; Felbeck *et al.,* 1984). To date, however, the latter have not yet been isolated either.

ACKNOWLEDGMENTS

We thank the pilots and crew of the research submersible ALVIN as well as the captain and crew of R/V ATLANTIS II and the "Angus" team for their efforts during the Galapagos Rift expedition, March 1985. Our work was supported by Grant No. OCE83-8631 of the National Science Foundation. The expedition was supported by a National Science Foundation grant OCE83-11256 to J. J. Childress and K. S. Johnson. S. Belkin is the recipient of a Dr. Chaim Weizmann Postdoctoral Fellowship for Scientific Research.

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SALINITY EFFECTS ON THE ACTIVITY OF GRANULAR HEMOCYTES OF AMERICAN OYSTERS, *CRASSOSTREA VIRGINICA*

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ABSTRACT

American oysters, *Crassostrea virginica,* from a high-salinity (HS) and a low-salinity (LS) location in the Chesapeake Bay were acclimated to six salinities (6–36 ppt) in the laboratory for 3–4 weeks. After acclimation, hemolymph was drawn from oysters and granular hemocytes were tested *in vitro.* Measurements of time to hemocyte spreading (TTS) and rate of hemocyte locomotion (ROL) were made in six media ranging in salinity from 6–36 ppt. TTS measurements were fastest at the acclimation salinities and slowed with acute rises in salinity. The time to spreading may be a measure of the osmotic adjustment process. Locomotion was dependent on ameboid shape. ROL decreased with acute rises in salinity for both populations, and increased with acute reductions in salinity for all test conditions except HS hemocytes acclimated at 30 and 36 ppt salinity. ROL tested at the acclimation salinities showed no differences between HS oysters (complete acclimation to lower salinities) but LS oyster hemocytes at 30 and 36 ppt were still slower even after 27 days of acclimation. There were a greater number of agranular hemocytes for HS oysters at all salinities. These findings are discussed in relation to osmotic adjustment, ameboid locomotion, acclimation, and disease susceptibility.

INTRODUCTION

Phagocytosis of foreign particles by hemolymph cells is a primary line of defense for American oysters, *Crassostrea virginica* (Cheng, 1975), and the bulk of phagocytic activity is performed by the granular hemocytes (Cheng, 1975; Foley and Cheng, 1975). Hence, proper function of the granular hemocytes may be required for defense against oyster diseases caused by parasites such as *Perkinsus marinum* (Mackin *et al.,* 1950; Perkins, 1969), *Haplosporidium costalis* (Wood and Andrews, 1962), and *H. nelsoni* (Haskin *et al.,* 1966; Sprague, 1978). A link between these diseases, which are commonly called Perkinsus (or Dermo), SSO, and MSX, respectively, is that each occurs as a chronic infestation only in high salinity regions (Mackin *et al.,* 1950; Mackin, 1955; Wood and Andrews, 1962; Andrews, 1964; Haskin and Ford, 1982). In addition, experimental work has demonstrated that established MSX infections can be rejected by oysters when they are transferred from high to low salinities (Sprague *et al.,* 1969; Haskin and Ford, 1982). One possible explanation for this pattern is that high salinity reduces the oysters' defense capacity and leaves them more susceptible to pathogenic parasites.

The hemolymph of marine molluscs, including bivalves, follows the osmotic strength and ionic composition of the ambient water (Hand and Stickle, 1977; Shum-

Received 15 April 1985; accepted 25 November 1985.

Abbreviations: CMFW—calcium and magnesium free water; FCW—full complement water; HS and LS—high and low salinity oyster populations; TTS—time to hemocyte spreading; ROL—rate of hemocyte locomotion.

way, 1977; Gilles, 1979). Thus, the hemocytes are exposed to the full range of salinities, 6–36 ppt (Galtsoff, 1964), experienced by the oyster. The objective of this study was to determine the effect of salinity changes on specific functions of the granular hemocytes of *C. virginica.*

The characteristic activity of granular hemocytes withdrawn from oysters has been described by Bang (1961) and by Foley and Cheng (1972). Chelating agents, such as ethylenediamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA), are often used to inhibit adherence, aggregation, or transformation of locomotory cells. (Noble, 1970; Edds, 1977; Kanungo, 1982; Kenney *et al.,* 1972). Taylor and Condeelis (1979) noted that calcium chelating agents cause both mobile freshwater amoebae and echinoderm eggs to cease movement and to form spherical shapes. Similar techniques were used in this study to inhibit hemocyte activity prior to *in vitro* experimentation.

Measurement of the time required for transformation, introduced here for the first time, is the cell response time, *i.e.,* the time from stimulation to locomotion. This study also measures the rate of hemocyte locomotion. Locomotory activity of mammalian leucocytes has been described with a variety of techniques (Wilkinson *et al.,* 1982; Keller, 1983) and Tackle (1984) recently utilized the technique of Lackie and Burns (1983) to describe insect hemocyte locomotion.

Results indicate that increased salinity retards hemocyte activity in both acute and acclimated conditions. The oysters in this study were also used to investigate the effects of different acclimation salinities on the physiological scope for growth (Newell *et al.,* 1986), and the protein and carbohydrate content of their cell-free hemolymph (Fisher and Newell, in prep.).

MATERIALS AND METHODS

Oyster collection and acclimation

High-salinity oysters (HS) were collected from an oceanic location at Wachapreague, Virginia, and low-salinity oysters (LS) were dredged from the Tred Avon tributary of the Choptank River in the upper Chesapeake Bay. HS (mean dry weight ± S. D. = 1.50 ± 0.46 g) were collected on 21 May 1984 and LS (1.13 ± 0.31 g) had been collected one week earlier and held in flow-through water in our laboratory, directly across the Choptank River from the Tred Avon site. Salinities at Wachapreague ranged from 27–32 ppt during May while those in Tred Avon ranged from 6–8 ppt.

Oysters were initially held at field ambient 15°C, then raised to 21°C for the salinity adjustment and acclimation period. Salinity adjustments were made in 6 ppt stages after an interval of at least 3 days at each stage (Table I). Acclimation to 6, 12, 18, 24, 30, and 36 ppt salinity occurred in 6 separate aquaria held at 21°C with 12 oysters from each location at each salinity. Acclimation periods varied, but the total number of days in adjustment and acclimation was the same for oysters from each location at any given salinity (Table I).

Oysters were fed 500 ml algae twice a day (*Isochrysis aff. galbana* and *Thalassiosira pseudonana*) at concentrations about 3% of the oysters' dry weight per day. Fine silt (<63 μm) was also added to the algae to improve oyster feeding and the water was continuously aerated. Water was changed and salinity adjusted twice each week.

Solutions and withdrawal of hemolymph

Two concentrated stock solutions were made for these experiments. Full-complement water (FCW) consisted of salts added in the proportions that they would normally

TABLE I

Adjustment and acclimation schedule[1]

Acclimation salinity	Days at intermediate salinities		Days at acclimation salinity	
	HS	LS	HS	LS
6	12	0	32	44
12	9	0	26	35
18	5	5	19	19
24	0	8	29	21
30	0	11	38	27
36	0	14	41	27

[1] A schedule of the number of days that oysters at each acclimation salinity spent during salinity adjustment (intermediate salinities) and at the final acclimation salinity.

be found in sea water. This consisted of 0.034 g $NaHCO_3$, 12.23 g $MgSO_4 \cdot 7H_2O$, 8.99 g $MgCl_2 \cdot 6H_2O$, 2.61 g $CaCl_2 \cdot 2H_2O$, 1.30 g KCl, and 47.84 g NaCl per liter of deionized H_2O. This made an artificial sea water equivalent to 60 ppt salinity which was diluted with distilled water to the test salinity (osmotic pressure measured by "Osmette A", Precision Systems, Inc.). Calcium- and magnesium-free water (CMFW) excluded calcium and magnesium ($MgSO_4$, $MgCl_2$, and $CaCl_2$) and sulfate requirements were met by the addition of 7.04 gl^{-1} Na_2SO_4. This solution was also diluted to the appropriate salinities. Tris buffer (10 m*M*) was added to both FCW and CMFW after dilution and the pH adjusted to 7.40 ± .03 with HCl.

A 3 ml syringe was loaded with 1.5 ml CMFW + 2% EDTA (59.5 m*M* EDTA). The CMFW was diluted to the oyster acclimation salinity (=CMFW-iso, or FCW-iso for FCW adjusted to the oyster acclimation salinity). Oyster valves were notched on the posterior side and fluid in the mantle cavity was drained. The syringe needle (22G, 1–½") was inserted into the adductor muscle and hemolymph was slowly drawn into a 1:1 dilution with the CMFW-iso + 2% EDTA. The syringe was gently rocked three-times to mix the contents without creating hemocyte clusters and one drop was added to each side of a Neubauer hemocytometer for hemocyte counts (Stein, 1975). The syringe was gently rocked 5–10 more times to allow aggregation of hemocytes into small clusters.

Hemocyte function tests

Within 15 seconds of withdrawal, 2–3 drops of the diluted hemolymph were added to the wells of a plastic tissue culture dish which had been pre-loaded with 1 ml CMFW-iso. The hemocytes were allowed to settle for 5 minutes, then EDTA was washed from the medium three times by slowly withdrawing 0.9 ml from the well and replacing it with CMFW-iso. The final removal of medium left the hemocytes adhering to the bottom of the well covered by 0.1 ml CMFW-iso. The timed portion of the experiment began when FCW was added at salinities of 6, 12, 18, 24, 30, or 36 ppt. The hemocytes in each well were examined with a Nikon inverted microscope (200×) after 5, 10, 15, 30, 45, 60, 90, and 120 minutes. At each examination, hemocyte clusters were checked for ameboid cells forming along the periphery and starting to migrate from the cluster. The sample was marked positive (=TTS) when the majority of clusters had ameboid cells along the entire periphery. Hemocytes that did not spread by 120 minutes were assigned the value of 150 minutes because after this time the hemocytes would sometimes disaggregate without distinct spreading or locomotion.

The rate of locomotion (ROL) was measured for 10–20 ameboid granular hemocytes in selected wells. A video camera was used to project the microscope image onto a television screen and the paths of the hemocytes were traced for 5 minutes on clear plastic overlayed on the screen. Most tracings were made between 60 and 150 minutes after the experiment began and readings of less than 0.75 μm/min were discounted as non-mobile cells.

Statistical evaluation

A Student-Newman-Kuels test (SNK) was used to determine differences between means for TTS measurements. Analysis of variance (ANOVA) was used to determine collection site, acclimation salinity, and test salinity differences. Linear regression was employed to determine the slopes of ROL measurements *versus* test salinity by acclimation salinity and location (origin). Those acclimation salinities for each location that exhibited a slope significantly different from zero were pooled for analysis of covariance and a regression equation was determined from the pooled data.

RESULTS

Hemocytes drawn into a 1:1 dilution with CMFW + 2% EDTA were spherical and non-mobile, but were not inhibited from adhering to each other and forming clusters on contact. The cells, whether single or in clusters, remained spherical and non-mobile during and after washing with CMFW. Only the addition of calcium and magnesium (as FCW) initiated spreading and locomotion by the hemocytes. Figure 1 shows the transformation and migration of granular hemocytes after the addition of FCW. EDTA was necessary because withdrawal of hemocytes in CMFW alone was not sufficient to keep all hemocytes spherical. Preliminary experiments had shown that withdrawal into CMFW + 2% EDTA, washing, and then adding FCW created a slightly longer TTS than withdrawal directly into FCW, but the additional steps had no measurable effect on ROL.

Time to hemocyte spreading (TTS)

Full-complement water, containing calcium and magnesium, was required for the initiation of cell spreading and subsequent mobility. There were two TTS responses to acute changes in salinity (Fig. 2). The most dramatic was a large increase in TTS when hemocytes from oysters acclimated to low salinities were raised to higher salinities. Greater differences between the acclimation and test salinity resulted in longer times to cell spreading. Hemocytes acclimated to 6 ppt and tested *in vitro* at 36 ppt, did not become ameboid after 120 minutes and were assigned the (conservative) value of 150 minutes. The second response to acute salinity change was a slight increase in TTS when hemocytes from oysters acclimated to high salinities were tested at the lowest test salinities. The shortest TTS resulted from hemocytes tested at or near their acclimation salinity and there were no significant differences in TTS among hemocytes tested and acclimated at the same salinities (circled points in Fig. 2). Oysters from the HS location had a faster TTS response than those from the LS location (ANOVA, $P < .05$).

Rate of hemocyte locomotion (ROL)

Granular hemocytes were not mobile when they were spherical, and the presence of calcium and magnesium was necessary for their transformation to an ameboid

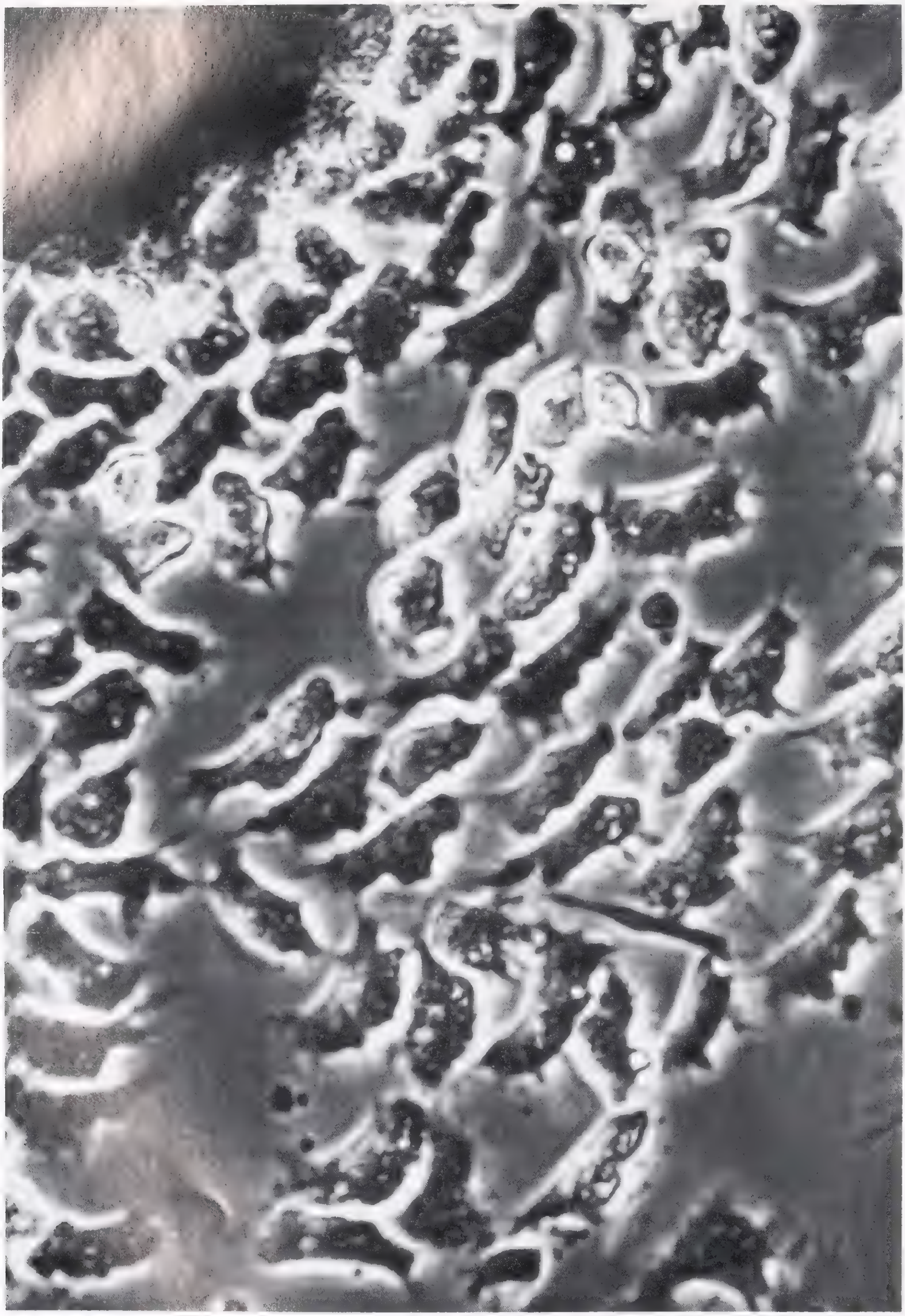

FIGURE 1. Oyster hemocytes on the periphery of a hemocyte cluster transform from a spherical to an ameboid shape (cell spreading) and migrate away from the cluster (locomotion). The crescent shape with the convex portion of the cell leading is typical for the locomotory hemocytes.

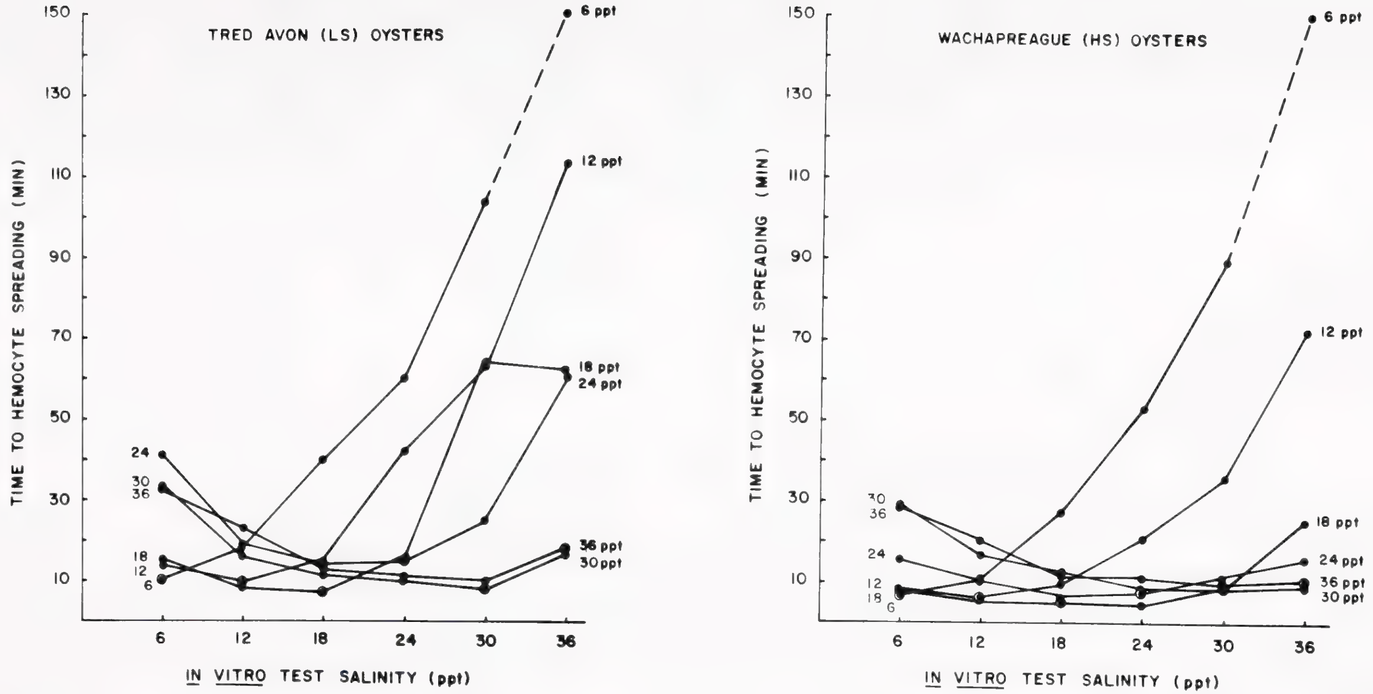

FIGURE 2. Average time to hemocyte spreading for oysters collected from a low-salinity (LS) and a high-salinity (HS) location and tested in a range (6–36 ppt) of salinities. Results from oysters at each acclimation salinity are presented separately. Dashed lines represent results obtained from assigned values (see text) and circled points represent measurements where the *in vitro* test salinity was the same as the acclimation salinity. Standard errors are presented in Table II.

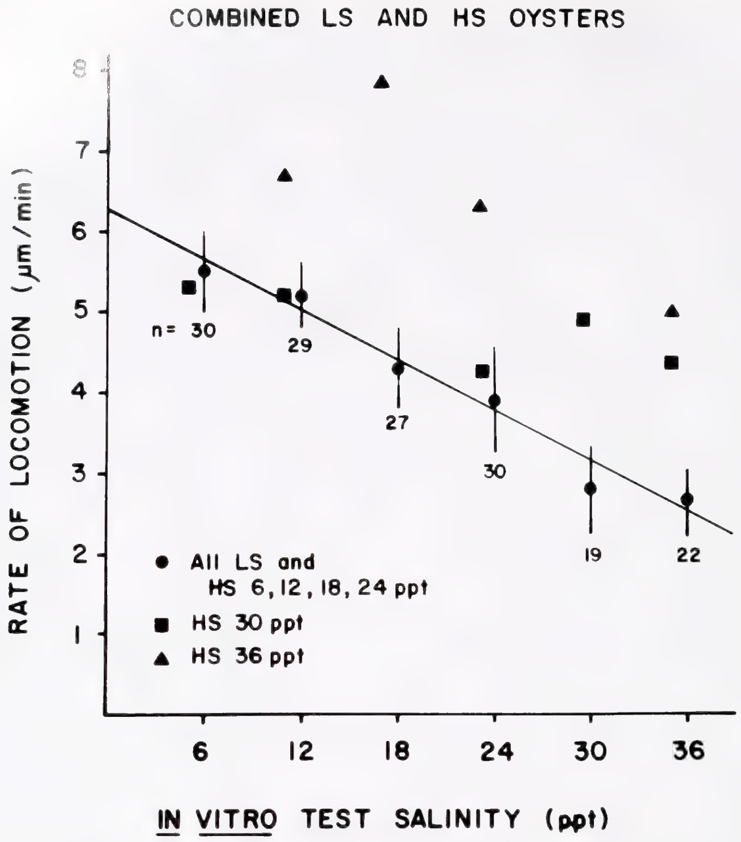

FIGURE 3. Average rates of hemocyte locomotion (ROL) for oysters collected from both locations and tested in a range (6–36 ppt) of salinities. Results from the high-salinity oysters acclimated to 30 and 36 ppt salinity (HS-30 and HS-36) did not exhibit slopes different from zero and are presented separately. All other oysters at all acclimation salinities from both collection sites showed slopes significantly different from zero and were pooled for presentation. There were no differences in the pooled data (ANCOVA, $P > .05$) and error bars show 95% confidence limits.

shape. Acute salinity changes affected the ROL of oyster hemocytes from both locations at nearly all acclimation salinities. That is, the ROL at all acclimation salinities from both locations, except HS oysters acclimated at 30 and 36 ppt, had a slope significantly different from zero when ROL was plotted *versus* test salinity (linear regression, $P < .05$). The results are presented in Figure 3, where the regression means and 95% confidence limits were pooled for data with no differences (ANOVA, $P > .05$), which excluded only HS-30 and HS-36. The regression is described by the equation

$$\text{ROL } (\mu\text{m/min}) = 6.25\text{–}0.104 \text{ test salinity (ppt)}$$

It is emphasized that this relationship holds for all LS oysters but only for HS oysters acclimated at 6–24 ppt.

There was a significant decrease in ROL for LS hemocytes acclimated and tested at 30 and 36 ppt (Fig. 4; SNK, $P < .05$). Thus, after 27 days of acclimation to high salinity, LS oyster hemocytes were still slower in high-salinity conditions than HS hemocytes. HS hemocytes showed no differences in ROL when measured at different

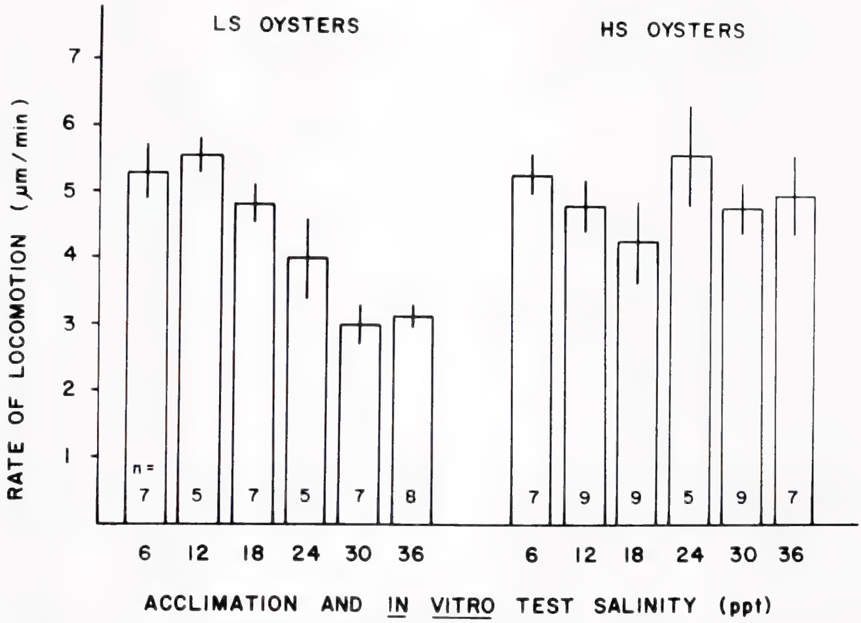

FIGURE 4. Average rates of hemocyte locomotion and standard errors for LS and HS of oysters in conditions where the *in vitro* test salinity was the same as the acclimation salinity. The periods of acclimation at each salinity ranged from 19–44 days (Table I). The LS oysters acclimated and tested at 30 and 36 ppt salinity were significantly slower than LS oysters from lower acclimation salinities while there were no differences between HS oysters from any acclimation salinity.

acclimation salinities (Fig. 4; ANOVA, $P > 0.05$). Hemocytes of oysters from both locations held and tested at their respective ambient salinities were not different (Fig. 4). The acute and acclimated TTS and ROL tests for both locations are compared in Table II. There were dramatic differences in TTS for both locations. TTS responses for LS oysters in higher salinities and for HS oysters in lower salinities had returned to ambient values after the acclimation period. ROL measurements for HS oysters showed no effect due to acute salinity reductions, and this did not change during the acclimation period. ROL measurements for LS oysters showed that hemocytes from 18 and 24 ppt salinities were partially returned to ambient rates, whereas hemocytes from 30 and 36 ppt salinities remained at the same reduced rates that were caused by the acute salinity increase.

Hemocytometer counts showed that there was no difference in the number of circulating granular hemocytes in the hemolymph of oysters from the two collection sites (Fig. 5), but there were greater numbers of circulating agranular hemocytes in the HS oysters at all acclimation salinities (ANOVA, $P < 0.05$).

DISCUSSION

Two major themes pursued by previous investigators have been the nutritive and defensive roles of oyster hemocytes (Feng *et al.,* 1977; Cheng, 1977). Phagocytosis is the major hemoctye activity that underlies these functions. Presumably, phagocytosis is influenced by the response-time of the hemocytes (TTS) and their subsequent locomotory ability (ROL). Phagocytosis may also be directly linked to locomotion by the process of endocytosis (Bretscher, 1984), by shared calcium-binding sites on the

TABLE II

Comparison of acute and acclimated conditions[1]

TTS: min (±S.E.)	Salinity 6	12	18	24	30	36
LS						
acute	†10.4 (0.5)	17.7 (3.6)	40.1 (4.3)	60.4 (8.4)	103.8 (10.2)	150.0 (0.0)
acclimated	†10.4 (0.5)	9.9 (1.0)	7.1 (3.5)	15.0 (4.7)	8.9 (2.4)	18.5 (3.0)
HS						
acute	28.9 (3.9)	17.0 (2.7)	12.7 (2.5)	8.4 (0.8)	†8.4 (0.8)	9.2 (0.6)
acclimated	7.6 (1.0	6.3 (1.7)	5.5 (0.5)	7.5 (1.4)	†8.4 (0.8)	10.9 (0.7)

ROL: μm min^{-1} (±S.E.)	Salinity 6	12	18	24	30	36
LS						
acute	†5.31 (.34)	5.00 (*)	3.38 (.38)	2.03 (.18)	3.13 (*)	2.51 (.18)
acclimated	†5.31 (.34)	5.56 (.26)	4.81 (.33)	4.02 (.61)	3.01 (.3)	3.16 (.17)
HS						
acute	5.25 (.31)	5.20 (.91)	4.38 (*)	4.26 (.35)	†4.83 (.41)	4.44 (.18)
acclimated	5.30 (.30)	4.83 (.39)	4.31 (.62)	5.58 (.77)	†4.83 (.41)	5.00 (.61)

[1] A comparison of time to hemocyte spreading (TTS) and rate of hemocyte locomotion (ROL) measurements in acute and acclimated conditions. Certain values (*) are estimated from the regression depicted in Figure 5. Acute data came from oysters in groups LS-6 and HS-30, the ambient salinities for Tred Avon and Wachapreague, respectively. Acclimated data came from tests at each acclimation salinity. Certain data (†) are repeated for acute and acclimated conditions.

cell membranes (Stockem and Klein, 1979), and by microfilament involvement (Spooner *et al.*, 1971; Axline and Reaven, 1974). Thus, TTS and ROL measure hemocyte characteristics fundamental to the phagocytic process. Although the effects of salinity on phagocytosis and overall defense must be further investigated, these hemocyte function tests may prove to be valuable indicators of the vitality of the oyster defense system.

The acute salinity tests revealed that hemocyte transformation was retarded by increased salinity (Fig. 2) and that greater retardation resulted from greater differences between acclimation and test salinities. This implies that the time period was being used by the hemocytes for osmotic adjustment. It is possible that hemocytes must be iso- or hyperosmotic to their environment in order to become ameboid. When *in vitro* measurements on oyster hemocytes were made at acclimation salinities, only 5–10 minutes were required for hemocyte spreading (circled points in Fig. 2). This may have been the fastest that spreading could occur, since no osmotic adjustment was necessary under such conditions.

The effect of salinity on hemocyte locomotion was evident in oysters from both locations (Fig. 5). As with TTS measurements, a greater salinity differential resulted in a greater change in hemocyte activity. Acute salinity increases retarded the activity of *C. virginica* hemocytes and acute salinity decreases enhanced hemocyte activities, implying that it is more time-consuming for oysters to adjust to higher salinities than to lower salinities. This also has been found in *Modiolus demissus,* where intracellular biochemical changes stabilize within hours in hypo-osmotic conditions (Pierce, 1970) but require weeks in hyperosmotic conditions (Baginski and Pierce, 1975). Several investigators have found that isolated cells respond more rapidly to resist swelling (hypo-osmotic conditions) than to resist shrinking (Davenport, 1982, for review).

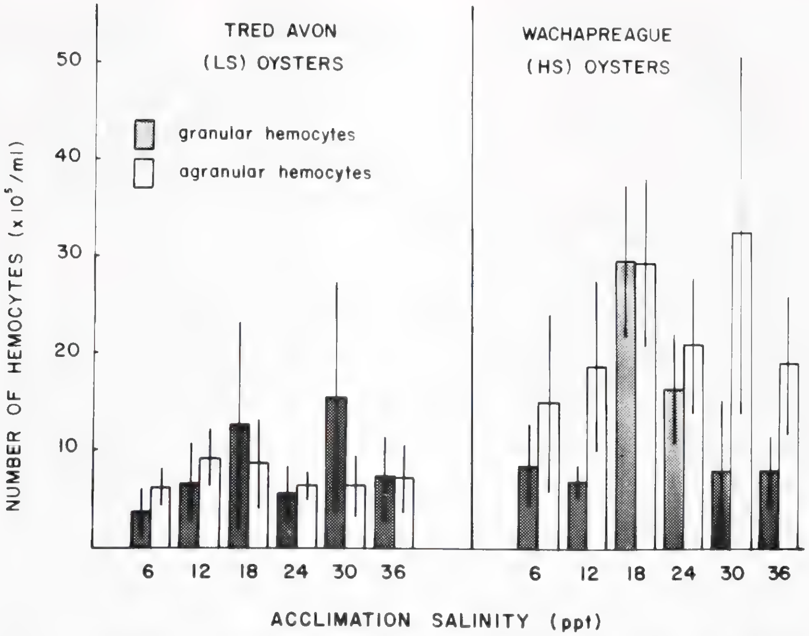

FIGURE 5. Average numbers and standard error bars for circulating granular and agranular oyster hemocytes for oysters collected from each location at each acclimation salinity. The number of agranular hemocytes is greater for oysters collected from a high-salinity (HS) location over all acclimation salinities.

Wachapreague oysters (HS) acclimated at 30 and 36 ppt, by maintaining the same hemocyte ROL at all test salinities, were exceptions to the ROL pattern (Fig. 3). Yet this rate was equal to the maximum attained by hemocytes under any other condition. The enhancement effect of lowered salinity may have been masked by the fact that they were already mobile at a maximum rate. Enhancement with decreased salinity for the other HS oyster hemocytes might then be an artificial response, brought about by their acclimation to lower salinities.

Hemocytes from Tred Avon (LS) oysters maintained at their ambient salinity (LS-6) showed dramatic TTS and ROL responses to acute increases in salinity (Figs. 2, 3). Measurement of TTS after high-salinity acclimation revealed that hemocyte transformation was no longer retarded by high salinity, *i.e.*, they were completely acclimated. Hemocyte locomotion (ROL), however, showed only partial acclimation to 18 and 24 ppt salinity, and was still retarded by 30 and 36 ppt salinity (Table II, Fig. 4). Acclimation by HS oysters is difficult to discern since the acute effect of decreased salinity was relatively minor in both TTS and ROL.

Since the retarded rate of locomotion for LS oysters in high salinities did not completely acclimate to ambient rates, it can be assumed that either the acclimation period was too short or that there were genetic differences between the LS and HS oysters. The latter possibility is reinforced by large differences in protein and carbohydrate concentrations in the cell-free plasma from the same oysters studied here (Fisher and Newell, in prep.), by differences in hemocyte counts (Fig. 5), and by differences in feeding rate, oxygen consumption and scope for growth which were also measured from the same oysters studied here (Newell *et al.*, 1986). Yet, electrophoretic comparisons (Newell *et al.*, 1986) could determine no dissimilarities between the populations.

There is an apparent contradiction between the evidence which showed a retarding influence of high salinity on hemocytes and the measurements made on HS oysters at their ambient salinities. Comparison of TTS and ROL for HS oysters at 30 and 36 ppt salinity with LS oysters at 6 ppt, indicates that high salinity does not have a detrimental effect in the field. A possible explanation for this discrepancy is that HS oysters have found a means to compensate for the high salinity through adaptation or long-term acclimation. High-salinity oysters in Delaware Bay that have increased their resistance to the MSX parasite through laboratory selection experiments (Haskin and Ford, 1979) may be an example of such an adaptation.

High-salinity susceptibility to diseases may be a consequence of such factors as intolerance of low salinities by the parasites (Haskin and Ford, 1982) and the downstream force and dilution of infective particles by fresh water (Mackin, 1955; Haskin and Ford, 1982). These results imply that salinity effects on the oyster defense system may also be a factor. For example, a decrease in hemocyte activity may have abetted the greater susceptibility to MSX shown by low-salinity oysters transplanted in Delaware Bay (Haskin and Ford, 1979). Similarly, the enhancement of hemocyte locomotion in reduced salinities may have aided in the expulsion of MSX from oysters transplanted to low salinity (Sprague *et al.,* 1969; Haskin and Ford, 1982).

The types or numbers of hemocytes available may also play a role in defense. Hemocyte counts in drawn hemolymph (Fig. 5) reflect only the circulating hemocyte level and it is unlikely that these circulating levels are an accurate estimate of the total hemocyte count (Feng, 1965). It is interesting however, that there is a greater number of circulating agranular hemocytes in HS oysters than in LS oysters. Farley (1968) found that these agranular hemocytes were present in much greater number in MSX-infected oysters. Only one oyster in this study was found to be infected with MSX and that animal had circulating granular and agranular hemocyte counts average for its group.

Measurement of hemocyte activity can aid in understanding cellular defense processes in bivalves. In this study, hemocyte measurements yielded sensitive and specific responses to salinity changes. Hemocyte spreading may be linked to intracellular volume regulation and the salinity effects on hemocyte locomotion may have illustrated a fundamental characteristic of ameboid locomotion. In addition, differences in hemocyte activities found between the low- and high-salinity collection sites raise questions concerning the long-term effects of adaptation to different salinities. Since the HS oysters were survivors in a location with heavy disease pressure, it is intriguing to speculate whether their ability to maintain maximal hemocyte activity in high salinities provided them with greater disease resistance.

ACKNOWLEDGMENTS

We wish to thank Jay Nair, Terry Paulson, and Anna McGinn for their patient technical assistance, Mr. Fred Kern and Austin Farley for their histological examination of the oysters, and Mike Castagna for obtaining oysters from Wachapreague. We also wish to thank Drs. Susan Ford and Chris Bayne for their constructive criticism of this manuscript. This work was funded (in part) by the Maryland Sea Grant College under grants from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U. S. Department of Commerce, and from the State of Maryland, Federal grant NA-84AA-D-00014. The U. S. Government is authorized to produce and distribute reprints for governmental purposes, notwithstanding any copyright notation that may appear hereon.

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ISOLATION AND SEQUENCE ANALYSIS OF A PIGMENT-DISPERSING HORMONE FROM EYESTALKS OF THE CRAB, *CANCER MAGISTER*

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ABSTRACT

The application of separation procedures, including liquid partition, gel filtration, ion-exchange chromatography, partition chromatography, and reversed-phase high-performance liquid chromatography, enabled the purification of an octadecapeptide pigment-dispersing hormone (PDH) from powdered lyophilized eyestalks of the crab, *Cancer magister.* Automated Edman degradation, followed by the identification of carboxyl-terminal amide, established the sequence of the peptide as: Asn-Ser-Glu-Leu-Ile-Asn-Ser-Ile-Leu-Gly-Leu-Pro-Lys-Val-Met-Asn-Asp-Ala-NH_2. The characterized *Cancer* PDH is structurally identical to an octadecapeptide PDH of *Uca pugilator,* but differs from the PDH of *Pandalus borealis* at positions 3, 4, 11, 13, 16, and 17.

INTRODUCTION

Reversible pigment movements within integumental chromatophores and light/dark-adaptive pigment migration in certain ommatidial cells of crustaceans are controlled by neurosecretory hormones (reviews: Kleinholz, 1961; Fingerman, 1970; Rao, 1985). Until recently, knowledge of the primary structure of crustacean pigmentary-effector hormones was restricted to two hormones (Kleinholz, 1985): red pigment concentrating hormone (RPCH), an octapeptide (Fernlund and Josefsson, 1972; Fernlund, 1974a, b), and light-adapting distal retinal pigment hormone (DRPH), an octadecapeptide, isolated from eyestalks of *Pandalus borealis* (Fernlund, 1971, 1976, 1977). The DRPH is one of the several forms of a molecule that acts both as a light-adapting hormone and chromatophorotropic pigment-dispersing hormone, PDH (Kleinholz, 1970, 1975; Riehm and Rao, 1982; Josefsson, 1983).

The structural basis of the heterogeneity of PDH (=DRPH) within any given species remains unknown, but progress is being made in evaluating the structural relationships among the PDHs from different species (Newcomb, 1983; Keller and Kegel, 1984; Quackenbush and Fingerman, 1984; Newcomb *et al.,* 1985). Recently Rao *et al.* (1985) determined the primary structure of an octadecapeptide PDH isolated from eyestalks of the fiddler crab *Uca pugilator.* The sequenced *Uca* PDH shares the amino-terminal and carboxyl-terminal residues, as well as a hexapeptide core sequence, with *Pandalus* PDH (=DRPH: Fernlund, 1976), but shows residue substitutions at six positions. As part of our continuing efforts to characterize the PDHs of representative

Received 9 September 1985; accepted 25 November 1985.

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Abbreviations: PDH, pigment-dispersing hormone (also called DRPH, light-adapting distal retinal pigment hormone); RPCH, red pigment concentrating hormone; TDE, thiodiethanol.

species of Crustacea, this report deals with the purification and sequence analysis of the major form of PDH from eyestalks of the crab *Cancer magister*.

MATERIALS AND METHODS

Eyestalks and animals

Eyestalks, freshly removed from male *Cancer magister* and frozen at −20°C, were supplied by a dealer at whose plant the living crabs were prepared for market. Upon arrival at the laboratory the eyestalks were lyophilized and stored at −20°C in tightly stoppered plastic bottles. Specimens of *Uca pugilator,* utilized in bioassays, were collected at Panacea, Florida, or obtained through a supplier from Panama City, Florida.

Initial extraction and partition

The initial extractions, with all solutions containing 0.1% of thiodiethanol (TDE), were done as described by Fernlund (1971). Powdered lyophilized eyestalks (100 g, about 1250 eyestalks) were heated to near boiling for 5 min with 99% ethanol:0.1 *M* NH_3, 700:300, v/v; the filter cake was washed three times with 200 to 300 ml portions of the same solvent. The combined filtrates, after reduction to about 50 ml in a rotary evaporator, were diluted with water to 100 g and were extracted four times with 100 ml portions of the upper phase of n-butanol-0.1 *M* NH_3, 1:1, v/v. The combined butanol phases, taken to near dryness by rotary evaporation, were dissolved in 0.2 *M* HCl and then partitioned with cyclohexane-butanol, 4:1, v/v (Fernlund, 1971). The HCl phase was dried in a rotary evaporator.

In scaled-up extractions, 250 g of powdered eyestalks were mixed with 900 ml of boiling distilled water and heated for 10 min in a boiling water bath. The mixture was cooled in an ice-water bath and under running tap water to room temperature. Glacial HOAc was added slowly and with stirring to pH 5, and 1 ml of TDE added. The suspension was centrifuged, and the residue extracted six times after stirring for 1 to 3 hours each with 250 ml portions of 0.5 *M* HOAc. The combined supernatants were divided into two portions, each was reduced in volume by rotary evaporation and lyophilized. The lyophilized residues were then subjected to Fernlund's extraction described above, starting with (unheated) 99% ethanol-0.1 *M* NH_3 and proceeding through separation of the HCl phase.

Chromatographic purification

The details of chromatographic conditions are given in the legends to figures and an outline of the protocol is presented here. The dried HCl phase (obtained as above) was extracted with aliquots of 1 *M* HOAc, centrifuged to remove any sediment, and filtered through a column of Sephadex G-25 fine with 1 *M* HOAc containing 0.1% TDE as the eluant. The PDH zones from two preparations filtered on Sephadex G-25 were combined and subjected to cation exchange chromatography on CM Sephadex C-25 utilizing ammonium acetate buffers (containing 0.02% TDE). Accumulated TDE was removed from the combined fractions constituting the major peak of PDH activity by filtering the dissolved residues on columns of Sephadex G-25-superfine, with 0.02 *M* ammonium bicarbonate containing 0.02% TDE as eluant, instead of heating to 50°C under vacuum as described by Fernlund (1971). The PDH zones processed as above from several preparations (from 1.181 kg of eyestalks) were pooled and further purified by partition chromatography on Sephadex G-50-superfine and by reversed-phase high performance liquid chromatography (HPLC) on a Whatman Partisil-10 ODS-2 column as described by Rao *et al.* (1985).

Amino acid composition and sequence analysis

Peptide samples were hydrolyzed *in vacuo* with constant boiling HCl for 22 h at 110°C, and analyzed by Beckman 119CL Analyzer. Sequencing analysis was carried out by stepwise Edman degradation, using a gas-phase automated sequencer (Applied Biosystems, Model 470A), coupled with HPLC identification of phenylthiohydantoin (PTH)-amino acids. Identification of the carboxyl-terminal residue as alanine was followed by a study to determine whether it occurs as Ala-NH_2. For this purpose 1.68 nmol of purified PDH was treated with 0.2% trifluoroacetic acid for 3 h in a boiling water bath (Tarr, 1985), so as to cleave peptide bonds involving Asp (Asp-Ala here). Then the sample was dried, reacted with phenylisothiocyanate, and compared by HPLC with authentic PTC (phenylthiocarbamyl)-Ala and PTC-Ala-NH_2.

Synthetic peptides

Synthetic preparations of *Pandalus* PDH (=DRPH; Riehm and Rao, 1982) and *Uca* PDH (Rao *et al.*, 1985) served as reference compounds for comparison by HPLC or by bioassay.

Bioassay

Solutions of purified and synthetic peptides, as well as aliquots of the fractions from purifications, were assayed for melanophore pigment dispersion in destalked (eyestalkless) fiddler crabs. Samples to be assayed were dissolved in physiological saline (Pantin, 1934). Unless otherwise stated, the injected volume was 50 μl/crab. The stages of melanophore pigment distribution were examined microscopically and scored according to the five-point scale of Hogben and Slome (1931). The observed pigment-dispersing responses were quantified in terms of activity values, as described by Kleinholz and Kimball (1965) and Fingerman *et al.* (1967).

RESULTS

When the HCl fraction of eyestalk extract, resulting from initial extraction and partition, was filtered through a column of Sephadex G-25 fine, the PDH zone emerged at about 0.53 column volume (Fig. 1). Upon CM-Sephadex chromatography several peaks of PDH activity could be resolved, of which the major peak (fractions 26–33) eluted at about one column volume (Fig. 2) and accounted for 66% of the recovered activity. When this material was purified by partition chromatography on a column of Sephadex G-50 superfine, two zones of PDH activity were evident (Fig. 3). The major zone (eluting at 0.74 column volume) was further purified by reversed-phase HPLC (Fig. 4). In this chromatography PDH activity was mainly associated with the UV-peak having a retention time of 14.5 min. Synthetic PDH of *Uca* displayed an identical retention time (14.5 min), whereas *Pandalus* PDH emerged slightly earlier, 13.8 min (Fig. 4).

Quantitative amino acid analysis of a sample of the HPLC-purified PDH indicated that the yield from 1.181 kg of eyestalk powder (about 14,750 eyestalks from *Cancer magister*) was 12.2 nmol (24 μg). This accounted for about 4% of the original activity as assayed by melanophore pigment dispersion in destalked *Uca*.

Amino acid analysis (1.87 nmol sample) showed that the purified PDH of *Cancer* has 18 residues: Asx, 3.61 (4); Ser, 2.03 (2); Glx, 1.31 (1); Pro, 1.36 (1); Gly, 1.44 (1); Ala, 1.32 (1); Val, 1.04 (1); Met, 0.84 (1); Ile, 1.56 (2); Leu, 2.49 (3); Lys, 0.93 (1).

Automated gas phase sequencing (1.68 nmol sample) enabled the assignment of the sequence of residues 1 to 18 (Fig. 5). Since cycle 19 was blank, Ala^{18} can be

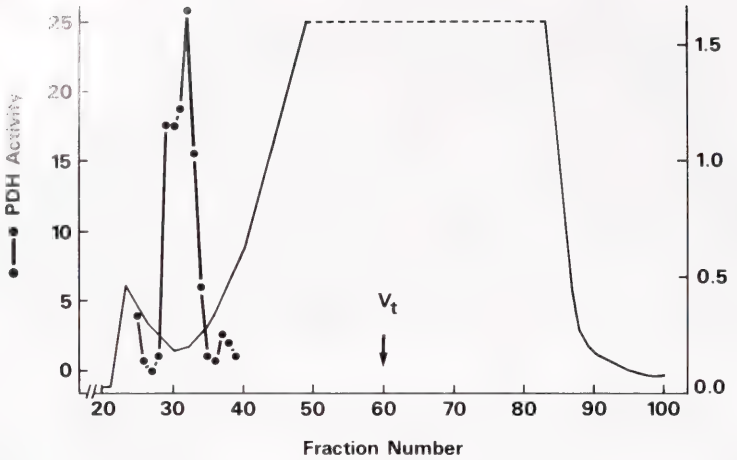

FIGURE 1. Gel filtration of extract of 100 g (1250 eyestalks) of *Cancer magister* on Sephadex G-25 fine. Initial extraction and partition procedures were as described by Fernlund (1971). Column: 2.5 × 92.7 cm; eluant: 1 M HOAc; flow rate: 44.4 ml/h; fraction size: 7.4 ml; V_t: total column volume, 448 ml. Bioassay: 5 μl of a fraction were mixed with 200 μl of Pantin's saline and 25 μl of the mixture was injected into each of 3 destalked *Uca pugilator*. The PDH effect is expressed as activity values (Kleinholz and Kimball, 1965).

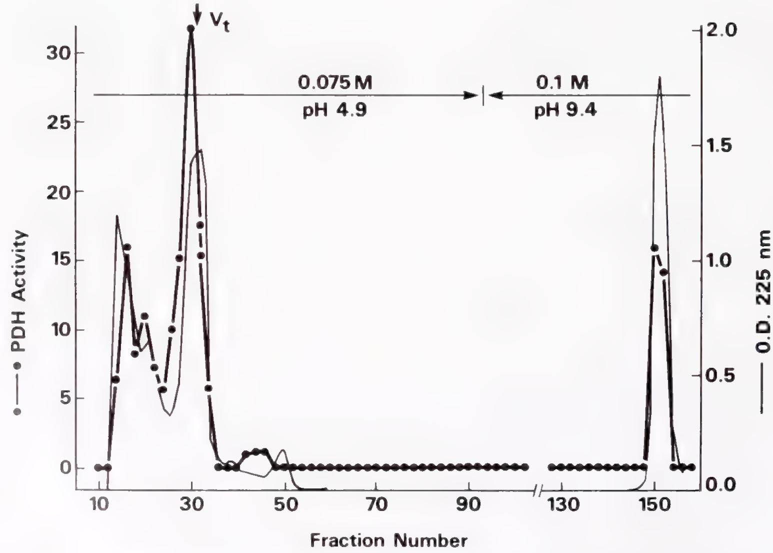

FIGURE 2. Cation-exchange chromatography on CM Sephadex C-25 of the PDH zones (fractions 29–33, Fig. 1) from two preparations filtered on G-25 Sephadex (extract of 223 g of lyophilized eyestalk powder). Column: 0.9 × 106 cm; V_t: total column volume, 67.5 ml; eluant: 0.075 *M*, pH 4.9 ammonium acetate; fractions of 2.8 ml were collected every 12.7 min to fraction 92; the head of buffer above the gel was then replaced with 0.1 *M*, pH 9.4 ammonium acetate and step-wise elution with this buffer continued, fractions of 2.8 ml being taken every 12.7 min. Bioassay: aliquots of 20 μl from alternate fractions from the column were lyophilized, the residue dissolved in 350 μl Pantin's saline, and injected into 4 destalked *Uca* (50 μl/crab). The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967).

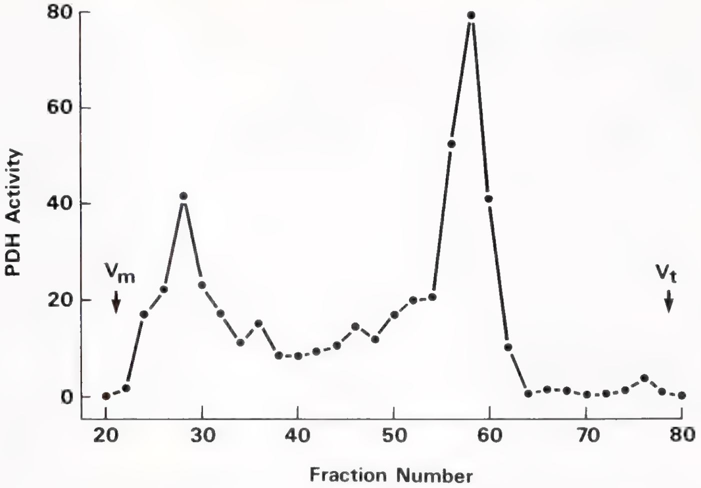

FIGURE 3. Partition chromatography on Sephadex G-50 superfine of the combined major PDH zones obtained by cation-exchange chromatography (fractions 26–33 in Fig. 2) from 1.181 kg of eyestalk powder. Column: 1.5 × 88.5 cm; eluant (without TDE): organic phase of n-butanol-HOAc-H_2O, 4:1:5, v/v; flow rate of 3.9 ml/h, fraction size 2.0 ml; V_m, volume of mobile phase, 44.5 ml; V_t, total volume of column, 156 ml. Bioassay: 5 µl aliquots from alternate fractions were lyophilized, the residues dissolved in 0.35 ml saline, and 50 µl injected into each of 3 test *Uca*. The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967).

regarded as the carboxyl-terminal residue. Further characterization of this residue, by HPLC analysis of the products of dilute acid cleavage, showed that the carboxyl-terminal Ala occurs in an amidated form. Thus the primary structure of the purified PDH from *Cancer magister* is: Asn-Ser-Glu-Leu-Ile-Asn-Ser-Ile-Leu-Gly-Leu-Pro-Lys-Val-Met-Asn-Asp-Ala-NH_2. A synthetic preparation of this octadecapeptide (Rao *et al.*, 1985) and the purified native PDH from *Cancer magister* were equipotent in assays for melanophore pigment dispersion in destalked *Uca pugilator* (Fig. 6).

DISCUSSION

The present study shows that the PDH purified from eyestalks of *Cancer magister* is structurally identical to the major form of PDH in eyestalks of *Uca pugilator* (Rao *et al.*, 1985). For convenience in discussion this octadecapeptide will be hereafter called β-PDH, whereas the first characterized PDH (=DRPH) from *Pandalus borealis* (Fernlund, 1976) will be designated as α-PDH. The amino acid sequences of the two PDHs show 66.7% identity, the differences being found at positions 3, 4, 11, 13, 16, and 17, as shown below:

α-PDH: $\overset{1}{\text{A}}$sn-Ser-$\overset{3}{\text{G}}$ly-Met-$\overset{5}{\text{I}}$le-Asn-$\overset{7}{\text{S}}$er-Ile-$\overset{9}{\text{L}}$eu-Gly-$\overset{11}{\text{I}}$le-Pro-$\overset{13}{\text{A}}$rg-Val-$\overset{15}{\text{M}}$et-Thr-$\overset{17}{\text{G}}$lu-Ala-NH_2

β-PDH: Asn-Ser-*Glu*-*Leu*-Ile-Asn-Ser-Ile-Leu-Gly-*Leu*-Pro-*Lys*-Val-Met-*Asn*-*Asp*-Ala-NH_2

Besides sharing the amino and carboxyl-terminal residues, the two PDHs have a common hexapeptide core sequence (residues 5–10: Ile-Asn-Ser-Ile-Leu-Gly). Whether these residues comprise the "message sequence" of the PDH remains unknown, although studies with synthetic truncated analogs point to the importance of residues 6–9 for PDH activity (Riehm and Rao, 1982; Riehm *et al.*, 1985).

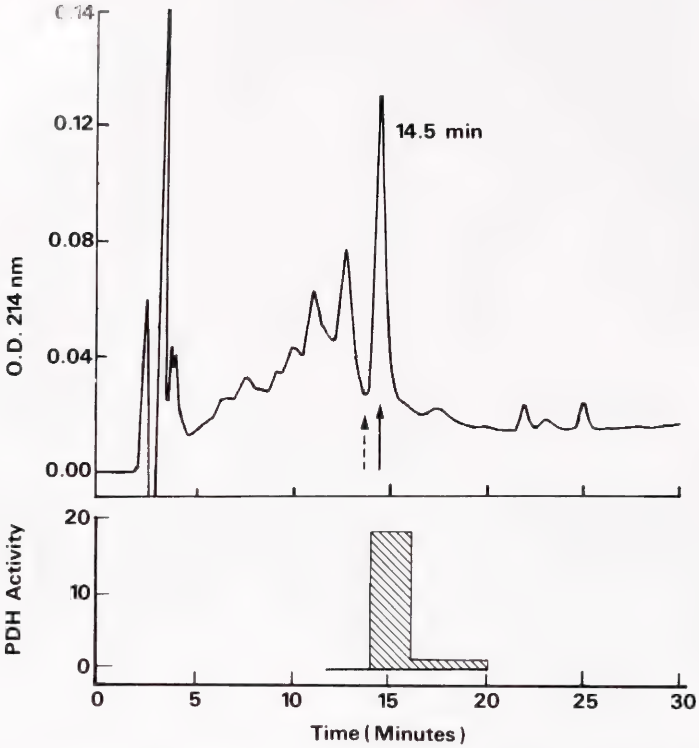

FIGURE 4. The major active zone from partition chromatography (fractions 55–60, Fig. 3) was subjected to reversed-phase HPLC on a column of Partisil-10 ODS-2 (4.6 mm × 25 cm, Whatman) utilizing Waters Liquid Chromatography System (Data Module 730; Programmable Controller 721; Pumps 510; Absorbance Detector 441). Solvent system: linear gradient elution for 30 min from 30% CH_3CN to 45% CH_3CN, both with 0.1% trifluoroacetic acid; flow rate, 1 ml/min. Bioassays were done with 0.05% of each fraction; each aliquot was lyophilized and dissolved in 0.5 ml saline; injected 50 μl/crab into 5 test *Uca*. The reference compounds utilized were: synthetic PDH of *Pandalus borealis* (Riehm and Rao, 1982), retention time 13.8 min (broken arrow); and synthetic PDH of *Uca* (Rao *et al.*, 1985), retention time 14.5 min (solid arrow). The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967).

Studies involving paper electrophoresis (Stephens *et al.*, 1956; Fingerman, 1966), disc electrophoresis (Rao *et al.*, 1970; Keller, 1977), ion-exchange chromatography (Rao *et al.*, 1970; Fernlund, 1971; Kleinholz, 1970, 1972; Dores and Herman, 1980), and reversed-phase HPLC (Keller and Kegel, 1984) showed that multiple forms of PDH are present in extracts of eyestalks or sinus glands of diverse decapod Crustacea. Initial resolution of the multiple forms of PDH can be best achieved, however, by ion-exchange chromatography (Kleinholz, 1970; Rao *et al.*, 1985). Since the PDHs separable by ion-exchange chromatography display identical elution profiles in gel filtration, the multiple forms of PDH are thought to differ in net charge but not in molecular size (Kleinholz, 1972). This view is supported by the finding that the two sequenced PDHs are both octadecapeptides that differ in net charge. The possibility that some of the variants are products of amino-terminal truncation, resulting from enzymatic breakdown, merits exploration.

The presence of Glu^3 (rather than Gly^3) renders β-PDH more acidic than α-PDH (Rao *et al.*, 1985). Consequently, during cation-exchange chromatography in low ionic strength buffers at pH 4.8–4.9, β-PDH elutes relatively fast, at about one column volume (Dores and Herman, 1980; Rao *et al.*, 1985), whereas α-PDH emerges later,

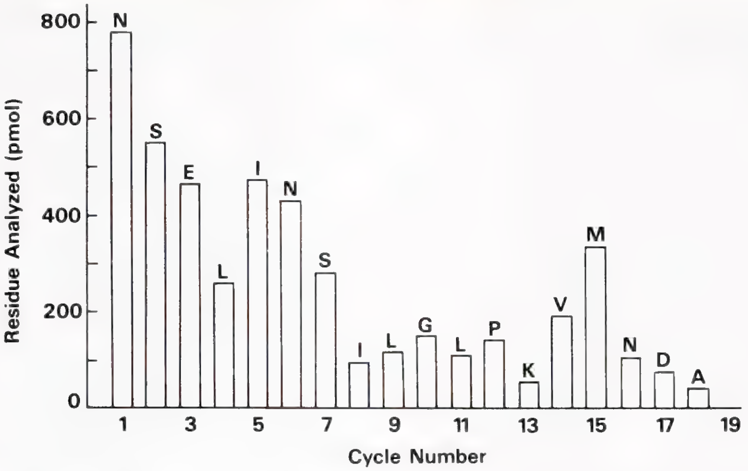

FIGURE 5. Yields of amino acid residues during the sequence analysis of *Cancer* PDH (1.68 nmol load) by stepwise Edman degradation, utilizing a gas-phase automated sequencer (Model 470 A, Applied Biosystems), coupled with HPLC identification of the resulting PTH-amino acids. Nomenclature of amino acids by the one-letter code: A (Ala), D (Asp), E (Glu), G (Gly), I (Ile), K (Lys), L (Leu), M (Met), N (Asn), P (Pro), S (Ser), V (Val).

at about three column volumes (Fernlund, 1971). In extracts of eyestalks from *Uca pugilator*, β-PDH is the major form of dispersing hormone (Rao *et al.*, 1985). A peak corresponding to α-PDH is either not evident (Rao *et al.*, 1985) or may account for

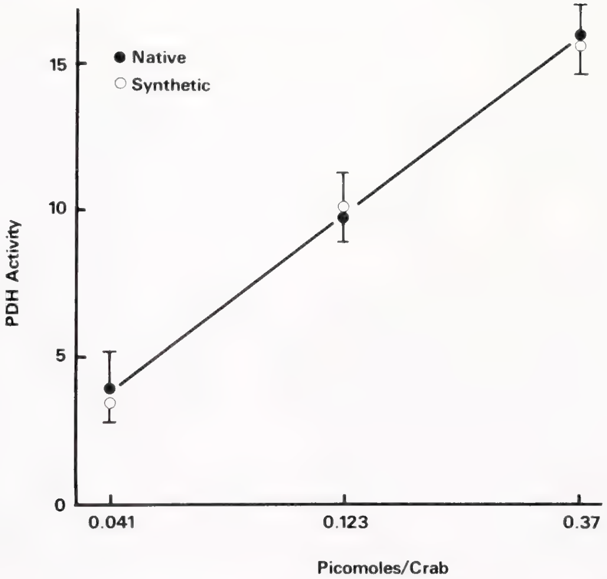

FIGURE 6. Dose-related melanophore pigment-dispersing activity elicited by purified native PDH of *Cancer magister* and a synthetic preparation of this peptide (first called *Uca* PDH; Rao *et al.*, 1985). Each data point (mean, standard deviation) was derived from 3 assays (5 destalked *Uca* per assay). The PDH activity is expressed as Standard Integrated Response values, as defined by Fingerman *et al.* (1967). Pantin's physiological saline (control) did not elicit any dispersion in the melanophores.

<2% of the recovered *Uca* PDH activity in cation-exchange chromatography (Dores and Herman, 1980). β-PDH is the predominant form in *Cancer magister* as well. In eyestalks of *Pandalus borealis* (Fernlund, 1971), however, α-PDH was reported to be the major form. Although two smaller peaks of activity eluting faster than α-PDH in cation-exchange chromatography were found in this species, it is not known whether either of these is identical to β-PDH. Clearly, much remains to be revealed about the structural basis of PDH heterogeneity within a given species and about the molecular interrelationships of PDHs among diverse species.

Although the two sequenced PDHs are both octadecapeptides with 67% identity in primary structure, the occurrence of markedly different PDHs has been reported. For example, Keller and Kegel (1984) found 28–29 amino acid residues in two PDHs purified by HPLC from extracts of sinus glands of the crab, *Eriocheir sinensis.* Quackenbush and Fingerman (1984) isolated a smaller PDH (1386 daltons) with arginine as the amino-terminal residue from eyestalks of *Uca pugilator.* Studies with PDH from sinus glands of *Cardisoma carnifex* have been inconclusive (Newcomb, 1983; Newcomb *et al.,* 1985), but they note that the peptide may have a blocked amino-terminus (unpublished data cited by Newcomb *et al.,* 1985). Since none of these seemingly distinct PDHs has been sequenced, their relationship to α-PDH and β-PDH remains unclear. It is of comparative interest that variation in molecular size as well as differences in net charge are evident among the melanophore-stimulating hormones (MSHs) of vertebrates (Li, 1978; Baker, 1979; Hruby *et al.,* 1984).

Both variation in chain length and residue substitutions are evident among arthropod peptides related to crustacean RPCH (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2; Fernlund and Josefsson, 1972). Thus, the related AKH (adipokinetic hormone) from locust is a decapeptide (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2; Stone *et al.,* 1976), whereas the related myotropic/cardioacceleratory hormones from cockroach are octapeptides: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2 and pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2 (Scarborough *et al.,* 1984; Witten *et al.,* 1984).

ACKNOWLEDGMENTS

This work was supported by National Science Foundation Grant DCB-8314737 to K.R.R. and J.P.R., and by a visiting professorship at the University of West Florida to L.H.K.

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Volume 170 Number 2

THE
BIOLOGICAL BULLETIN

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APRIL, 1986

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Woods Hole, Mass.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $20.00. Subscription per volume (three issues), $50.00 ($100.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor, or Pamela Clapp, Assistant Editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

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ISSN 0006-3185

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C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

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THE

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PUBLISHED BY
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APRIL, 1986

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

Marine Biological Laboratory
LIBRARY
APR 29 1986
Woods Hole, Mass.

The Biological Bulletin is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $20.00. Subscription per volume (three issues), $50.00 ($100.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor, or Pamela Clapp, Assistant Editor, Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

The Biological Bulletin (ISSN 0006-3185)

Postmaster: Send address changes to The Biological Bulletin,
Marine Biological Laboratory, Woods Hole, MA 02543.
Second-class postage paid at Woods Hole, MA, and additional mailing offices.

LANCASTER PRESS, INC., LANCASTER, PA

ERRATUM

THE BIOLOGICAL BULLETIN, Volume **168** (Supplement), Number 3, page 90.

The following correction should be made in the final paragraph of page 90 in the article by Dr. W. D. Russell-Hunter, "An evolutionary century at Woods Hole: instruction in invertebrate zoology" (1985, *Biol. Bull.* **168** (Suppl.): 88–98). The name T. W. Painter (found in the second sentence) should read T. S. Painter. As a matter of record, Dr. Painter was an instructor in the MBL's Invertebrate Zoology course during the years 1914–1916.

Reference: *Biol. Bull.* **170:** 145–175. (April, 1986)

SEXUAL PHEROMONES IN ALGAE

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I. INTRODUCTION

In 1972, L. Machlis compiled a review on sexual hormones in the plant kingdom. He listed four organisms, three fungi and one alga, in which substances had been identified at that time. At about the same time, antheridiogen A_{An} was characterized as a factor inducing sex organs in fern gametophytes (Nakanishi *et al.*, 1971). Machlis described this situation as the "coming of age of sex hormones in plants." Now, more than a decade later, significant progress has been achieved in some fields, but recognition of pheromonal factors in plant reproduction is still limited, judged by the almost complete lack of attention in recent monographic treatments such as Linskens and Heslop-Harrison (1984), or Hummel and Miller (1984).

Received 28 October 1985; accepted 3 January 1986.

Reviews on sexual interactions and cellular recognition in plants have been published by van den Ende (1976) and Heslop-Harrison (1978). They still represent the present state of knowledge in many species of algae. This review concentrates on two groups of algae in which significant progress has been achieved within the past few years: colonial green algae and marine Phaeophyceae.

The term "pheromone" was introduced by Karlson and Lüscher (1959) to characterize substances that coordinate activities between individuals within one species. Such substances can be subsumed under the term "hormone" in its broadest definition, but it is convenient to refer to diffusible substances travelling through the external media of air or water by using the more specific term "pheromone." Occasionally the term "gamone" is still in use. It was introduced by Hartmann and Schartau (1939) for all substances involved in sexual reproduction, including membrane-bound recognition factors. The very broad and highly diverging usage of the term "gamone" has proved impractical and confusing and therefore many authors prefer to discontinue its use.

Living organisms are open systems that depend on energy and nutrient uptake from the environment. One consequence of this dependency is that organisms have developed highly sensitive antennae for detecting signals from the outside world, including chemical factors originating from other organisms. Such chemical interactions between organisms have long been known and an elaborate set of descriptive terms has developed. In this context, sexual pheromones in plants represent but a very small selection of phenomena. Whittaker and Feeny (1971) have categorized interactions between organisms by chemicals which at least temporarily are part of the extra-organismic world. External media are either water or air, and messenger substances have to be water-soluble or volatile according to their distribution mechanisms. Figure 1 lists some terms used to describe chemical interactions between organisms, focusing on sexual pheromones in plants.

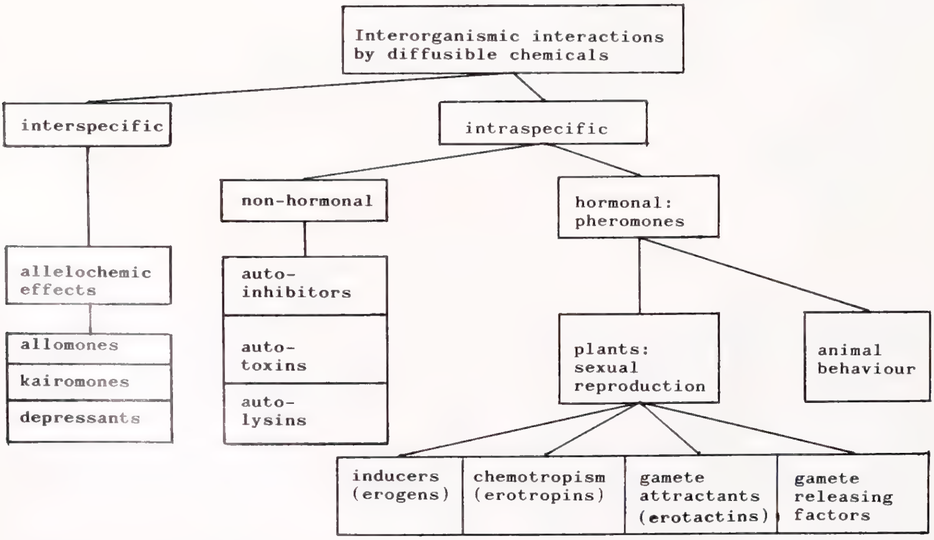

Interorganismic interactions by diffusible chemicals
interspecific
intraspecific
non-hormonal
hormonal: pheromones
allelochemic effects
allomones
kairomones
depressants
auto-inhibitors
auto-toxins
auto-lysins
plants: sexual reproduction
animal behaviour
inducers (erogens)
chemotropism (erotropins)
gamete attractants (erotactins)
gamete releasing factors

FIGURE 1. Synopsis of various possibilities for organisms to interact through diffusible chemicals.

Sexuality, defined as exchange and rearrangement of genes between individuals, is found in all groups of organisms from viruses to mammals and land plants and presumably has evolved independently in various groups. In eucaryotes however, basic sexual processes are rather uniform and include syngamy and meiosis, fusion, and rearrangement of nuclear genes.

In many groups sexual processes are closely connected with reproduction. Thus, in most animals and in many groups of plants the origination of new individuals is unseparably tied to sexuality, and any new individual is the immediate product of the fusion of two sexually differentiated cells (gametes). However, exceptions are found throughout the plant kingdom: new individuals are produced without sexuality in many groups, and survival strategies may rely on sexuality merely as one option under specific external conditions. Nevertheless plants have developed various strategies to support the encounter of gametes, including cooperation with animals as pollinators.

Attraction between gametes of different sex is a common phenomenon in lower plants (Altman and Dittmer, 1973). Some reports date back to the discovery of fertilization and sexual reproduction in organisms (Thuret, 1854; Pringsheim, 1855). In contrast, only few cases of sperm attraction are documented in animals, especially some marine invertebrates (Miller, 1979; Carré and Sardet, 1981).

II. Sexual Pheromones in Green Algae

II.1. Volvox

Species of the green algal genus *Volvox* are adapted to live in temporary freshwater pools. Thick-walled zygotes formed in sexual reproduction act as desiccation-resistant resting stages. Therefore timing and coordination of cellular activities in sexual reproduction is of vital necessity for survival of the populations. Field studies indicate synchronous activity changes in natural populations. Powers (1908), discussing *Volvox* populations in Nebraska, commented: "*Volvox* is able to appear, multiply and riot in sexual reproduction in pools of rain water of scarcely a fortnight's duration." The physiological basis of such phenomena has now been discovered in laboratory studies: chemical factors have been isolated and characterized which induce and synchronize sexual reproduction in *Volvox* populations.

II.1.1. *Low-molecular inducer in* Volvox capensis

V. capensis has been isolated from South Africa. New individuals develop from specialized cells (gonidia) inside the parent spheroid and are set free by enzymatic breakdown of the parent's matrix. In laboratory cultures, sexual spheroids appear under precisely predictable circumstances: if one single vegetative spheroid is introduced into 10 ml of medium, the first and second generations increase to 15 and 225 vegetative spheroids respectively. The next generation results in about 5000 individuals, most of which are sexual (Starr *et al.*, 1980). *V. capensis* is monoecious, *i.e.*, eggs and sperm packets are formed within the same spheroid. Variations of this experiment indicated that the factor which is responsible for the switch from vegetative to sexual reproduction coincides with the growing density of the cultures, and that it builds up gradually within a few generations. Active preparations could be obtained by enzymatic degradation of the gelatinous matrices of vegetative spheroids, which consist of glycoproteins. Active preparations were also obtained from hydrolysates of various other proteins. Eventually, L-glutamic acid was identified as the active factor in *Volvox capensis* with an activity at concentrations as low as 68 n*M* (Starr *et al.*, 1980).

When still inside the mother spheroid, embryos up to the 64 cell-stage respond to L-glutamic acid by forming eggs and sperm packets instead of vegetative gonidia. The build-up of L-glutamic acid in ageing populations of *Volvox capensis* originates from proteolytic degradation of the matrix in mother spheroids during release of daughter spheroids. The timing of this system seems to be well adapted: vegetative reproduction continues until individual numbers have increased by a factor of 5000. This is still in the logarithmic growth phase, before space and nutrition become limiting factors. Sexual reproduction then will produce, when fully successful, well over 10^4 zygotes from one individual, and thus offer a maximum chance of survival when the habitat eventually desiccates.

The molecular basis of the effect of L-glutamic acid on development in *V. capensis* is not known at present. It is active only in cultures that are kept in the light.

II.1.2. *High-molecular sex-inducers in* Volvox

In contrast to *Volvox capensis,* the following species of *Volvox* do not respond to L-glutamic acid: *V. aureus, V. carteri* f. *weismannia* and f. *nagariensis, V. gigas, V. rousselettii,* and *V. dissipatrix* (Jaenicke, 1982; Table I). Instead, species-specific high-molecular weight glycoproteins induce formation of sexual individuals. *Volvox carteri* f. *nagariensis* has been studied in great detail (Starr, 1970). This species has been isolated from Japan and India. It is heterothallic and dioecious. The female strain propagates without restriction by forming daughter individuals from gonidia (Fig. 2). Male cultures behave similarly, except that at regular intervals entire populations switch spontaneously to the formation of sexual individuals (Fig. 2) and subsequently collapse. A closer analysis showed that in male populations about one out of 20,000 individuals becomes "spontaneously" sexual. The presence of one single sexual male spheroid will divert the development of the entire next generation to sexual reproduction. One single male spheroid produces enough inducer activity to sexualize any other individual of the same species within a volume of 1 m^3 (Gilles *et al.,* 1984). In addition to this self-stimulation in male cultures, the factor induces formation of eggs in the next generation when added to vegetative female cultures (Fig. 2).

The inducing factor has been characterized as a glycoprotein (Kochert and Yates, 1974; Starr and Jaenicke, 1974). Although the molecular mechanism is not understood, the "first sexual male" occurs so reliably in ageing populations that most species of *Volvox* base their survival strategies on this phenomenon. Gilles *et al.* (1984) studied the effects of the inducer on development of *Volvox.* The molecule is not bound to cellular membranes of either vegetative cells or gonidia. Instead, highly active and

TABLE I

Characterization of sex-inducing pheromones in species of Volvox.

Species	50% activity g/liter	Chemical composition
V. capensis	10^{-6}	L-glutamate (in the light)
V. aureus	10^{-9}	protein, mol. wt. 200,000
V. carteri		
f. weismannii (America)	10^{-11}	glycoprotein, mol. wt. 30,000 80% protein, 20% carbohydrate
f. nagariensis (Japan, India)	3×10^{-12}	glycoprotein, mol. wt. 27,600 62% protein, 32% carbohydrate

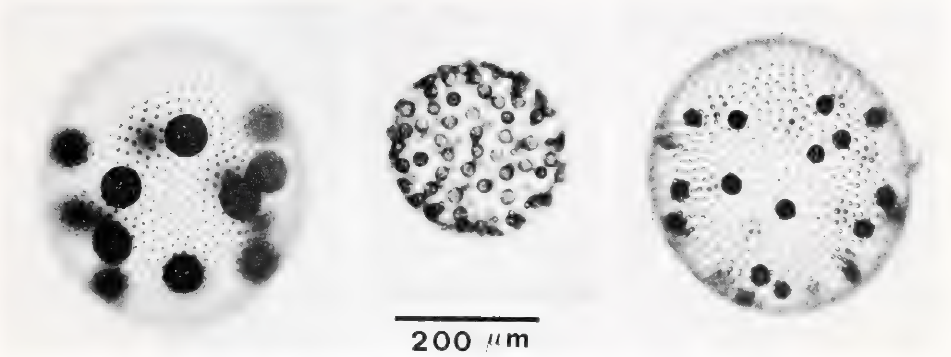


FIGURE 2. *Volvox carteri* f. *nagariensis*. Vegetative individual with young daughter colonies (left) and sexualized individuals with sperm packets (center) and eggs (right).

specific binding sites are localized in the extra-cellular matrix of the spheroids. Further studies indicated that the extra-cellular milieu of cyclic nucleotides, calmodulin, and Ca^{2+} has a very strong influence on the developmental pathway of the gonidia. Phosphodiesterase is known to interfere with this system, and has been found to mimic the inducer effect (Gilles *et al.*, 1983).

II.2. *Sexual reproduction in* Oedogonium

The genus *Oedogonium* contains filamentous freshwater green algae which may be monoecious or dioecious. Female plants differentiate oogonia from filament cells. Male gametophytes show two types of differentiation: macrandrous species form male gametes which are attracted to the oogonia. Nannandrous species produce motile androspores which settle near oogonia and germinate to few-celled dwarf plants. On these plants, antheridia release a few motile spermatozoids, which eventually fertilize the egg inside the oogonium. In the macrandrous species, *O. cardiacum*, sperm are attracted to the oogonium (Hoffman, 1960). More detailed experiments by Machlis *et al.* (1974) showed that the attractant is water-soluble and sensitive to high temperature or extreme pH. Its molecular weight was estimated to be between 500 and 1500.

Hormonal coordination in nannandrous species is more complex, and four stages can be distinguished (Rawitscher-Kunkel and Machlis, 1962):

—Attraction of androspores to oogonium initials.
—Oriented growth of dwarf male plants in relation to oogonia.
—The presence of dwarf male plants is necessary for complete differentiation of oogonia.
—Spermatozoids seem to be attracted to the oogonium aperture.

Although the molecular size and characteristics of the attractant in *Oedogonium cardiacum* suggest good potential for successful identification with modern analytical methods, no further studies have been reported on this fascinating system.

II.3. *Sexual chemotaxis in* Chlamydomonas

Tsubo (1961) reported chemotaxis of gametes in *C. moewusii* var. *rotunda*. "Minus" gametes of this species produced a volatile factor that attracted "plus" gametes. The factor was of low molecular weight and could be stripped from cultures by a stream of air. Chemotactic activity could also be detected in coal gas. The factor has not been identified. Later attempts to repeat Tsubo's observations failed because the original strains, although still maintained in culture collections, seem to have lost their sexual potential.

II.4. Closterium *factor*

Kato and Sasaki (1985) reported a pheromone system in the desmid *Closterium peracerosum-strigosum-littorale.* A diffusible glycoprotein secreted by the (−) mating type induces the formation of a lytic enzyme in the (+) strain, which results in the lysis of its cell walls and appearance of free protoplasts ready for sexual fusion and zygote formation. However, no details are known on active concentrations and function of the "protoplast releasing factor," and whether it acts as a lytic enzyme by itself.

II.5. *Sexual pheromones in Bacillariophyceae, Chrysophyceae, and Xanthophyceae*

Evidence of chemical factors coordinating mating activities is known for algae from several classes in addition to Chlorophyceae and Phaeophyceae. A few of these are discussed here, although none of the factors has been characterized.

Bacillariophyceae. The marine diatom *Rhabdonema adriaticum* has been studied by Rozumek (1968). It is dioecious and oogamous. Sexual stages are only formed when both mating types are placed in close vicinity in a culture dish. Attempts to isolate the active principle from culture filtrates failed.

Chrysophyceae. *Dinobryon cylindricum* is a freshwater alga forming colonies with loricate cells. The species is dioecious with motile male and sedentary female gametes. Sandgren (1981) demonstrated that female cultures secrete a factor which induces differentiation of gametes in male cultures. The factor diffuses through filters; no further details are known.

Xanthophyceae. Attraction of spermatozoids to oogonia is well known in monoecious species of *Vaucheria* (Oltmanns, 1922). This attraction can also be observed in dioecious species such as *V. litorea* (Fig. 3). First attempts to apply the isolation methods used for brown algal pheromones (Chapter III.2) were not successful (Müller, unpub.).

The Phaeophyceae and all cases mentioned here belong to the division Heterocontophyta. Algal pheromone systems are especially well represented in this group. C_8- and C_{11}-olefines which show pheromone-activity in marine Phaeophyceae have been repeatedly found in freshwater organisms (Jüttner and Müller, 1979), and lakes (Jüttner and Wurster, 1984), although their function or source remains obscure at present.

III. SEXUAL PHEROMONES IN BROWN ALGAE

III.1. *An overview*

Chemotaxis in brown algae has been known since Thuret's (1854) observations on sexual reproduction in the Fucaceae. Descriptions were given by Berthold (1881) and Oltmanns (1899) for gamete interaction in *Ectocarpus siliculosus* and by Kuckuck (1899) and Williams (1921) for chemotaxis in *Cutleria* and *Laminaria,* respectively. Thuret (1854) discussed an influence from *Fucus* eggs on the spermatozoids. In his words: *"Il est difficile, quand on observe ces phénomènes avec attention, de ne pas se laisser aller à croire qu'une impulsion particulière dirige les anthérozoides vers les corps qu'ils doivent féconder."*

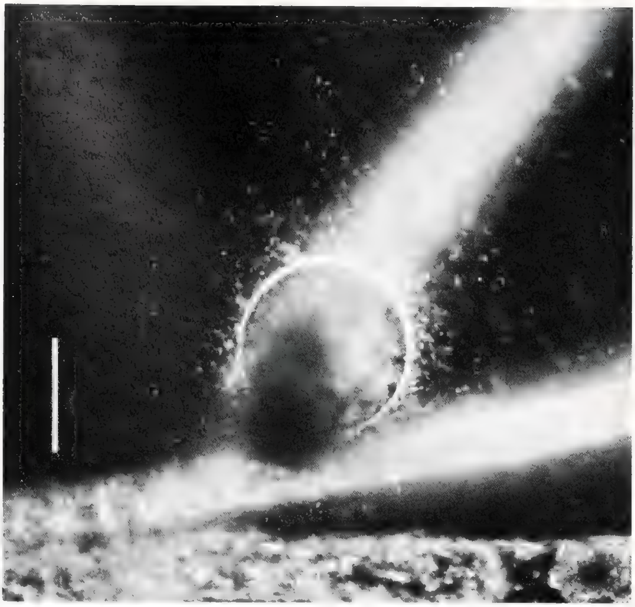


FIGURE 3. *Vaucheria litorea*. Spermatozoids attracted to oogonium. Scale bar 100 μm.

Progress in interpretation of these phenomena was hampered by the extremely small amounts of attractants secreted by female gametes. Their concentration was far below the potential of analytical chemistry until 20 years ago. The situation changed dramatically with the isolation and structural identification of the first brown algal pheromone, ectocarpene (Müller, 1967, 1968; Müller *et al.*, 1971; Jaenicke and Müller, 1973). In the past 17 years, 10 pheromone systems have been analyzed in detail, including all those species on which accurate observations on chemotaxis were depicted last century. The rapid progress in this field, documented in earlier review articles (Müller, 1972; Jaenicke, 1977; Müller, 1981; Jaenicke and Boland, 1982), was inspired and accelerated by cooperation between biologists and biochemists.

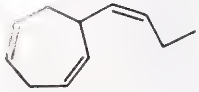
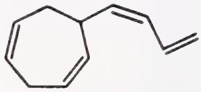
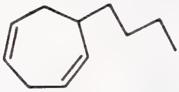
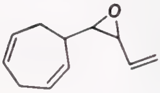
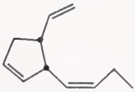
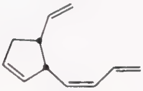
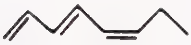
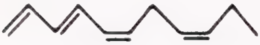
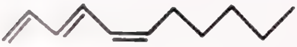
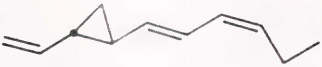
Today, our knowledge extends beyond information confined to the structures and sources of these compounds. Experimental results have made possible conclusions on pheromone biosynthesis, receptor specificity, details of the perception process and chiral recognition. Even interspecific interferences by chemical means in the natural habitat may be involved. It is a fascinating view of marine ecology to visualize the sea as the domain of such unusual messenger substances as these volatile, hydrophobic brown algal pheromones.

III.2. *Isolation and chemical structures*

Ectocarpene was the first brown algal pheromone identified. It was detected during culture studies with *Ectocarpus siliculosus* by its fragrant smell and a characteristic reaction (chemokinesis) of male gametes (Müller, 1967). To obtain the volatile active principle, a stream of purified air was first passed through suspensions of female gametes, then through a cold trap. In total, 92 mg pure pheromone could be collected from about 1 kg gametophytes (Müller *et al.*, 1971). The chemical structure of ectocarpene (Table II), was determined as (+)-(S)-6-(1Z-butenyl)-1,4-cycloheptadiene

TABLE II

Chemical structure of sexual hormones in brown algae

Structure	Trivial name	Taxa
1 	ectocarpene	*Ectocarpus siliculosus* *E. fasciculatus* *Sphacelaria rigidula* *Adenocystis utricularis*
2 	desmarestene	*Desmarestia aculeata* *D. viridis*
3 	dictyopterene C′	*Dictyota dichotoma*
4 	lamoxirene	Laminariaceae Alariaceae Lessoniaceae (29 species)
5 	multifidene	*Cutleria multifida* *Chorda tomentosa*
6 	viridiene	*Syringoderma phinneyi*
7 	fucoserratene	*Fucus serratus* *F. vesiculosus* *F. spiralis*
8 	finavarrene	*Ascophyllum nodosum* *Dictyosiphon foeniculaceus*
9 	cystophorene	*Cystophora siliquosa*
10 	hormosirene	*Hormosira banksii* *Xiphophora chondrophylla* *X. gladiata* *Durvillaea potatorum* *D. antarctica* *D. willana* *Scytosiphon lomentaria* *Colpomenia peregrina*

(Müller *et al.*, 1971; Jaenicke and Müller, 1973). It is identical with dictyopterene D′ in the essential oil from vegetative *Dictyopteris* thalli (Pettus and Moore, 1971; Kajiwara *et al.*, 1980a). Ectocarpene, the sexual pheromone of *Ectocarpus siliculosus*, is also secreted by female gametes and is a chemotactant in *Ectocarpus fasciculatus, Sphacelaria rigidula*, and *Adenocystis utricularis* (Müller and Gassmann, 1980; Müller *et al.*, 1985a).

Subsequently, two more sexual pheromones were identified using the same technique. In *Fucus serratus* and *F. vesiculosus*, prior to fertilization eggs secrete 1,3-trans,5-cis-octatriene (fucoserratene, Table II) which attracts spermatozoids (Müller and Jaenicke, 1973; Jaenicke and Seferiadis, 1975; Müller and Seferiadis, 1977). In *Cutleria multifida*, (+)-(3S,4S)-3((Z)-1-butenyl)-4-vinyl-cyclopentane (multifidene, Table II) is the specific male-attractant. Female gametes also produce ectocarpene and trans-4-(trans-1-propenyl)-5-vinylcyclohexene (aucantene) as by-products (Müller, 1974; Jaenicke *et al.*, 1974; Jaenicke and Boland, 1976; Boland *et al.*, 1983a; Paquette *et al.*, 1984).

Recent advances in extraction and analytical techniques resulted in structural identification of seven additional pheromones from brown algae in ten different orders (Tables II, III). All compounds known so far are volatile, lipophilic, more or less unsaturated C_8- or C_{11}-hydrocarbons with biogenetically related structures (Chapter III.3). To isolate the pheromones gamete secretions were enriched 10^5-fold using air circulation in a closed loop system with maximized exchange through the air-water interface (Grob and Zürcher, 1976; Müller *et al.*, 1979; Boland *et al.*, 1984b). Volatiles were adsorbed to a bed of 1.5 mg activated carbon (Fig. 4). After desorption with solvent, eluates were subjected to glass-capillary gas chromatography and GC-MS analysis.

Kajiwara *et al.* (1980b; 1981) found fucoserratene to be a constituent of receptacles of the fucoid seaweeds *Sargassum horneri, Fucus evanescens*, and *Pelvetia wrightii*. They have not shown, however, whether fucoserratene is actually secreted by eggs of these species. Therefore, and because of unresolved pheromone-receptor specificities as well as methodical drawbacks, the attempts of chemotaxis assays by Kajiwara *et al.* (1984) are of doubtful value. Nevertheless, it is likely that fucoserratene is indeed the native messenger in at least *F. evanescens*. It appears that closely related species or genera frequently have the same sexual pheromone. Recently, fucoserratene has been confirmed as the sperm attractant in *F. spiralis*, and although without evidence of biological activity, as egg secretion in *F. distichus* and *Hesperophycus harveyanus* (Müller and Gassmann, 1985).

Pelvetia fastigiata eggs produce 1(3E,5Z,8Z)-undecatetraene (finavarrene, Table II), and those of *Pelvetiopsis limitata* produce finavarrene, 1,3E,5Z-undecatriene and pentadecane as prominent compounds (Müller and Gassmann, 1985). Information on biological activity is also lacking in these species. Finavarrene was first reported as a constituent in vegetative thalli of *Dictyopteris* (Moore, 1977). It is a specific sperm attractant in *Ascophyllum nodosum* (Müller *et al.*, 1982a) and *Dictyosiphon foeniculaceus* (Peters and Müller, 1986). Finavarrene is also secreted by *Spermatochnus paradoxus* gametes, but no biological function could be detected in this species (Müller *et al.*, 1981b). In *Cystophora siliquosa* a similar, partially hydrogenated compound, 1,3E,5Z-undecatriene (cystophorene, Table II) has been identified as sexual pheromone (Müller *et al.*, 1985b).

Trans-1-vinyl-2(1E,3Z-hexadienyl)-cyclopropane (hormosirene, Table II) is a sperm attractant in *Hormosira banksii* (Müller *et al.*, 1984), *Durvillaea potatorum, D. antarctica, D. willana, Xiphophora chondrophylla, X. gladiata*, and two distantly related species, *Scytosiphon lomentaria* and *Colpomenia peregrina* (Müller *et al.*, 1985b). It

TABLE III

Taxonomic relationships of brown algal taxa with identified sexual pheromones

Order	Family	Species
Ectocarpales	Ectocarpaceae	*Ectocarpus siliculosus*
		E. fasciculatus
Cutleriales	Cutleriaceae	*Cutleria multifida*
Dictyotales	Dictyotaceae	*Dictyota dichotoma*
		Dictyopteris sp.*
Tilopteridales	Tilopteridaceae	*Haplospora globosa**
Sphacelariales	Sphacelariaceae	*Sphacelaria rigidula*
Syringodermatales	Syringodermataceae	*Syringoderma phinneyi*
Dictyosiphonales	Punctariaceae	*Adenocystis utricularis*
	Dictyosiphonaceae	*Dictyosiphon foeniculaceus*
Scytosiphonales	Scytosiphonaceae	*Scytosiphon lomentaria*
		Colpomenia peregrina
Chordariales	Spermatochnaceae	*Spermatochnus paradoxus**
Desmarestiales	Desmarestiaceae	*Desmarestia aculeata*
		D. viridis
Sporochnales	Sporochnaceae	*Perithalia caudata**
Laminariales	Chordaceae	*Chorda tomentosa*
		*C. filum**
	Phyllariaceae	*Saccorhiza polyschides**
		*S. dermatodea**
	Laminariaceae	*Laminaria* (7 species)
		Pleurophycus gardneri
		Agarum cribrosum
		Kjellmaniella gyrata
		Hedophyllum sessile
		Cymathere triplicata
	Alariaceae	*Alaria* (3 species)
		Undaria pinnatifida
		Pterygophora californica
		Eisenia arborea
		Ecklonia radiata
	Lessoniaceae	*Macrocystis pyrifera*
		M. integrifolia
		Nereocystis luetkeana
		Pelagophycus porra
		Dictyoneuropsis reticulata
		Lessoniopsis littoralis
		Lessonia variegata
Fucales	Fucaceae	*Fucus serratus*
		F. vesiculosus
		F. spiralis
		*F. evanescens**
		*F. distichus**
		Ascophyllum nodosum
		*Pelvetia wrightii**
		*P. fastigiata**
		*Pelvetiopsis limitata**
		*Hesperophycus harveyanus**
		Xiphophora chondrophylla
		X. gladiata
	Hormosiraceae	*Hormosira banksii*
	Cystoseiraceae	*Cystophora siliquosa*
	Sargassaceae	*Sargassum horneri**
Durvillaeales	Durvillaeaceae	*Durvillaea potatorum*
		D. antarctica
		D. willana

* Species with known secretions, but their biological activity not confirmed.

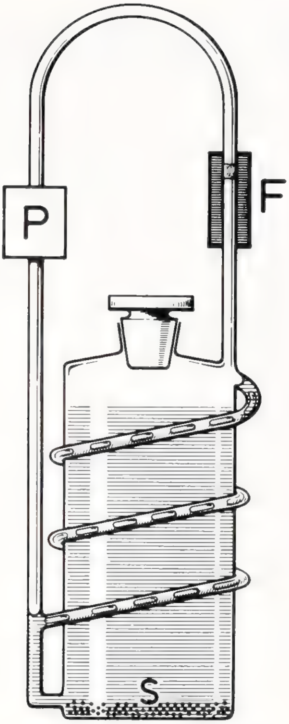


FIGURE 4. Extraction apparatus. Closed-loop stripping procedure after Grob and Zürcher (1976) adapted for seawater. P = adsorption filter consisting of 1.5 mg activated carbon. S = biological sample (eggs or fertile gametophytes).

has also been demonstrated in *Haplospora globosa,* but without relation to sexual reproduction (Kuhlenkamp and Müller, 1985). Hormosirene was known previously as dictyopterene B, a component of the *Dictyopteris* essential oil (Pettus and Moore, 1971; Kajiwara *et al.,* 1980a). In biosynthesis of cycloheptadiene pheromones, trans- (like hormosirene) and cis-disubstituted cyclopropanes are simultaneously formed intermediates. Ectocarpene is probably derived directly from the cis-disubstituted isomer of hormosirene (Pettus and Moore 1971; Moore, 1977; Boland and Mertes, 1985; Chapter III.3). Probably for this reason, hormosirene is normally found as a by-product in gamete secretions containing ectocarpene. The attractant of *Dictyota dichotoma,* (−)-(R)-6-butyl-cyclohepta-2,5-diene (Müller *et al.,* 1981a; Table II), is a side chain-hydrogenated derivative of ectocarpene. It is identical with dictyopterene C′, a minor constituent of the essential oil from vegetative *Dictyopteris* plants (Pettus and Moore, 1971; Kajiwara *et al.,* 1980a).

The main pheromone fraction secreted by female gametes of *Syringoderma phinneyi* has been identified as cis-3-(1Z,3-butadienyl)-4-vinylcyclopentene (viridiene, Table II) (Müller *et al.,* 1982c). Its absolute configuration (3R,4S) and the enantiomer composition of the natural pheromone was established by enantio- and stereospecific synthesis, HPLC separation of pheromone enantiomers and biological activity tests (Boland *et al.,* 1985).

In five species of Laminariales, Lüning and Müller (1978) observed triggering of explosive spermatozoid release (Chapter III.4) and the subsequent chemotactic response caused by a volatile factor. It was shown that eggs of *Laminaria digitata, L. hyperborea,* and *L. saccharina* produce ectocarpene, pentadecane, and a then unknown compound with the formula $C_{11}H_{14}O$, representing the biologically active principle (Müller *et al.*, 1979; Müller and Gassmann, 1980). It took six years before Marner *et al.* (1984) identified the pheromone, from the total amount of about 25 μg collected in this period. Its molecular structure is 1-(1′,2′-cis-epoxi-but-3′-enyl)-cyclohepta-2,5,-diene. An oxygen-containing compound was a novel feature among brown algal pheromones. Referring to its source and structure, the *Laminaria* sperm releasing and attracting substance was named lamoxirene (Table II).

Pheromone-mediated sperm release from antheridia has been observed in all members of Laminariales, Desmarestiales, and Sporochnales so far examined (see III.4). The specific activity of lamoxirene has been demonstrated in 26 species of Laminariales, representing members of three families: Laminariaceae, Alariaceae, and Lessoniaceae (Table III). Pheromone extracts in 11 species, including the economically important kelps *Macrocystis pyrifera, Laminaria japonica,* and *Undaria pinnatifida,* showed the same bouquet of lamoxirene and by-products. This means that the hormonal systems of these three families are indistinguishable (Maier, 1984; Müller and Maier, 1985).

In *Chorda filum,* one of the two species of the Chordaceae, the chemical structure of the sperm-releasing and -attracting pheromone is still unknown, but it is related to lamoxirene.

The pheromone bouquet of *Chorda tomentosa* (Fig. 5), a monoecious species, contains two pairs of compounds with a corresponding pattern of double-bonds and a common biogenetic origin (Chapter III.3): ectocarpene/multifidene and butyl-

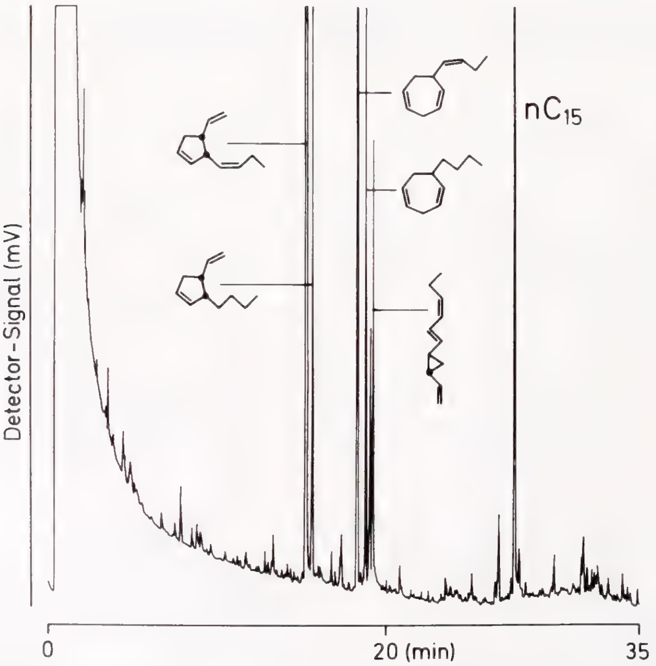


FIGURE 5. Gas chromatogram (glass capillary, SE 54i, on column injection) of an eluate from a suspension of *Chorda tomentosa* gametophytes, showing egg secretions.

cycloheptadiene/vinylbutyl-cyclopentene as well as hormosirene (Maier, 1984; Maier *et al.*, 1984). Using synthetic pheromones and analogous compounds as probes for pheromone receptor specificity in *Chorda tomentosa*, (+)-multifidene was identified as the biologically active messenger.

In *Saccorhiza dermatodea* and *S. polyschides* (Phyllariaceae), ectocarpene, also known from *Ectocarpus*, *Sphacelaria*, and *Adenocystis*, is the only prominent egg secretion.

Desmarestene (Table II) has been found as the sexual pheromone in *Desmarestia aculeata* and *D. viridis*. Ectocarpene and viridiene are prominent by-products (Müller *et al.*, 1982b).

In Sporochnales, studies on sexual pheromone specificity are presently in progress. As a first result, the sum formula of the pheromone in *Perithalia caudata* (Müller *et al.*, 1985c) seems to be identical with lamoxirene ($C_{11}H_{14}O$).

III.3. *Biosynthesis and secretion*

Brown algal pheromones can be classified in four groups relating to their molecular structure: (a) cyclopropanes, (b) cyclopentenes, (c) cycloheptadienes, and (d) acyclic olefines (Table IV). With the exception of fucoserratene (C_8), they are more or less unsaturated C_{11}-hydrocarbons. The pheromones lack methyl branches. In each class, the basic ring structure is the same. Another conservative structural feature, the vinyl group of the cyclopentenes, is integrated into the ring system in cycloheptadiene-pheromones. Modifications, mainly in the number of double bonds, are found only in the butyl-side chains (R, Table IV).

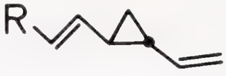
Joint occurrence of compounds with identical substitution, as, for example, hormosirene/ectocarpene/multifidene or desmarestene/viridiene in secretions from female gametes, give further indications of a common biosynthetic pathway. The presence and structure of minor by-products may also provide evidence on biosynthetic routes.

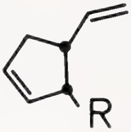
As yet, no experiments on pheromone biosynthesis have been attempted with brown algae. However, the South African composite *Senecio isatideus*, which produces ectocarpene in considerable amounts (Bohlmann *et al.*, 1979; Boland *et al.*, 1982a) can be used as a model system. A tentative biosynthetic scheme has been deduced from feeding experiments with cuttings of *Senecio* using tritium- and deuterium-labeled fatty acid precursors. According to the scheme proposed (Table V) brown algal pheromones can be derived in a common route from unsaturated fatty acids (Boland and Mertes, 1985). (^{3}H)dodeca-3,6,9,-trienoic acid is converted to labeled ectocarpene, whereas, for example, deuterated nona-3,6,-dienoic acid results in formation of fucoserratene, which is not originally present in the *Senecio* oil components (Boland and Mertes, 1985).

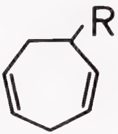
According to this hypothetical scheme, pheromone biosynthesis is initiated by abstraction of a hydrogen radical from a 1,4-pentadienyl-fragment of free polyunsaturated fatty acids (Table V). In contrast to plant lipoxygenase-catalysed reactions, in which addition of oxygen follows, the primary radical is oxidized to a carbonium ion. Stabilization takes place by loss of carbon dioxide and rearrangement of double bonds. If the abstraction of hydrogen occurs from the methyl group in 5-position of a fatty acid, the terminating vinyl group of an acyclic pheromone like fucoserratene or finavarrene may be formed. Pheromone biogenesis may be directed to disubstituted cyclopropanes and cyclopentenes by radical formation at position 8. Depending on the folding of the fatty acid on the enzyme(s), cyclization may result in cyclopropanyl- or cyclopentenyl-fatty acids. This is followed by double bond rearrangements and loss of carbon dioxide. Formation of cis- and trans-disubstituted cyclopropanes may be

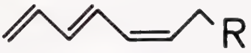
TABLE IV

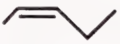
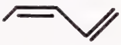
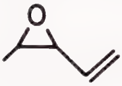
Structural classes of brown algal pheromones


(a)


(b)


(c)


(d)

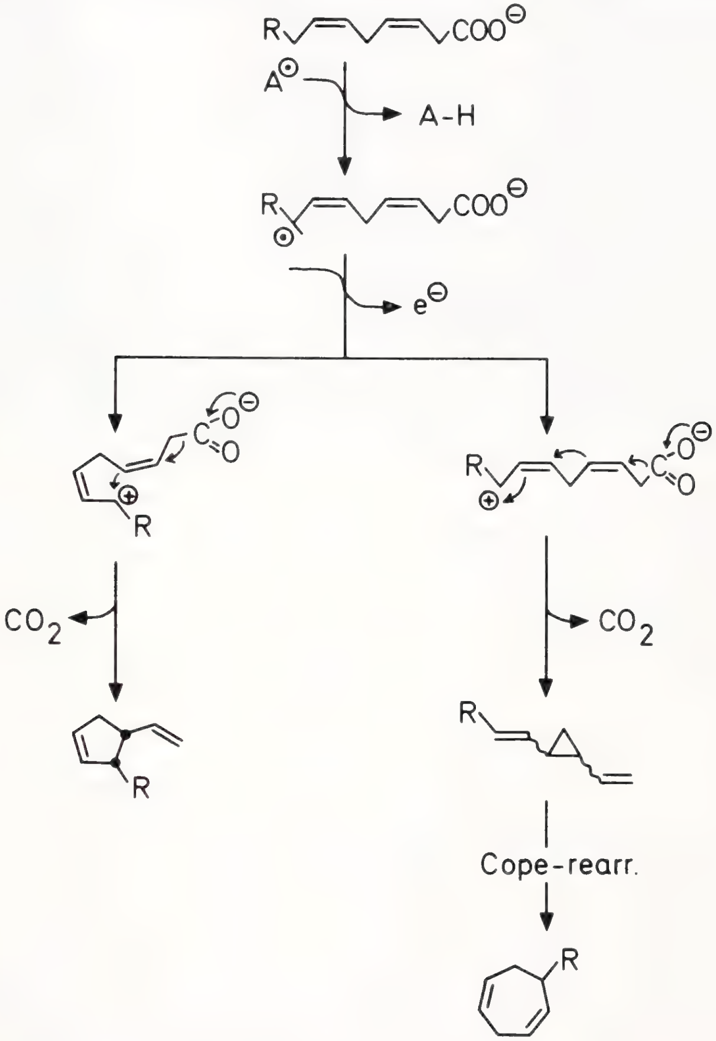
	(a)	(b)	(c)	(d)
$R = -CH_3$				●
$R = -C_4H_9$		●	●	●
R = 	●	●	●	●
R = 		●	●	
R = 			●	

possible. Cis-substituted cyclopropanes are unstable, even below room temperature, and undergo a rearrangement to 2,5-cycloheptadienes such as ectocarpene, whereas the trans-isomers occur in nature (for example, hormosirene). Consequently, the alkenyl substituent of cycloheptadiene systems arises from the aliphatic terminus of a given fatty acid, while the ring derives from the former carbonyl end (Boland and Mertes, 1985).

Judging by the chemotactic response of male gametes, pheromone secretion starts shortly after settling of motile female gametes or release of eggs in oogamous brown algae and ceases after sexual fusion. In Fucales and Laminariales, about 10^{-12}–10^{-14} moles of pheromone can be secreted per egg. For isogamous species, reliable data are not available. The site of pheromone biosynthesis and the cellular pathways up to secretion are unknown, but Clayton (1984a) described characteristic, probably dictyosome-derived vesicles with osmiophilic contents exclusively in female gametes of

TABLE V

Biosynthesis of alicyclic brown algal pheromones (R: see Table IV)



Scytosiphon. They were found close to and fused with the plasma membrane and it was suggested that they contain the pheromone. Allen and Brawley (1984) observed secretion of small vesicles (0.7 μm diameter) from unfertilized eggs of *Fucus vesiculosus* by video-enhanced light microscopy.

III.4. *Pheromone-mediated spermatozoid release*

In oogamous brown algae of the orders Laminariales, Desmarestiales, and Sporochnales a two-step chemical coordination in gamete interaction involves triggering of spermatozoid release prior to chemotactic orientation by the same pheromone. The messenger substance secreted by freshly released eggs causes explosive dehiscence of antheridia within a few seconds after perception (Lüning and Müller, 1978; Müller and Lüning, 1981; Müller *et al.*, 1982b; Maier, 1984; Maier *et al.*, 1984; Müller and Maier, 1985; Kajiwara *et al.*, 1985; Müller *et al.*, 1985c). Such a correlation between egg release and subsequent spermatozoid discharge has already been recognized by Tseng *et al.* (1959) and Norton and Burrows (1969) in *Laminaria japonica* and *Saccorhiza polyschides*, respectively. However, it was only in 1978 that the participation of low-molecular volatile pheromones was demonstrated in five species of Laminariales (Lüning and Müller, 1978), stimulating further research in this field.

Recently, joint efforts by biologists and chemists culminated in the structural identification of the release-triggering substances in *Desmarestia aculeata* and *D. viridis* (desmarestene), *Chorda tomentosa* [(+)-multifidene], and lamoxirene in *Laminaria digitata* and several other species of Laminariales (Müller *et al.*, 1982b; Maier *et al.*, 1984; Marner *et al.*, 1984; Müller and Maier, 1985). In *Desmarestia aculeata* and *D. viridis* the threshold concentration for spermatozoid release with racemic desmarestene was determined as 9×10^{-11} *M* (Müller *et al.*, 1982b). Racemic lamoxirene in concentrations as low as 1×10^{-11} *M* induces spermatozoid release in *Laminaria digitata, L. japonica, Macrocystis pyrifera,* and other species (Maier, 1984; Müller and Maier, 1985). In *Chorda tomentosa,* the threshold concentration is even lower at 1×10^{-12} *M* with (+)-multifidene (Maier *et al.*, 1984). This corresponds to the lowest effective concentration ever measured in brown algal pheromone systems. It can be estimated that in *Chorda tomentosa* no more than 600 signal molecules hit the cell surface within the reaction time of 20 seconds (Maier *et al.*, 1984).

Pheromone-mediated spermatozoid release is a unique system for the study of perception and transduction of chemical signals in plant cells. The pheromone-sensitive target cells, the antheridia, although rather small, can be easily identified and are freely accessible for experimental manipulation. To date there is no other example in plants where a known chemical messenger induces a comparably rapid reaction.

In *Laminaria digitata,* the cellular mechanism has been investigated in detail (Maier and Müller, 1982; Maier, 1982). During release, the antheridium opens by a circular rupture which removes the characteristic apical thickening of the cell wall (cap). The single spermatozoid is squeezed out, driven by internal pressure created by mucopolysaccharides (Figs. 6, 7). Osmotic phenomena are not involved, and participation of a specifically activated autolysine has been proposed. The spermatozoid is actively involved in its own release. In addition, the mechanism depends on certain extracellular cations, especially Na^+ and Ca^{2+} in at least two successive steps of the stimulus-reaction coupling. For example, Ca^{2+}-antagonists like La^{3+} and ruthenium red as well as verapamil, a calmodulin inhibitor, strongly affect pheromone action. The results obtained so far indicate that transient cation fluxes and Ca^{2+} as "second messenger" are involved, but definite proof is still lacking. After release, the response of spermatozoids to the same pheromone signal changes to chemotaxis, for which a different sensory system is certainly required.

III.5. *Chemotaxis*

III.5.1. *Approach mechanisms in male gametes*

Chemotactic responses of motile cells to chemical gradients may involve several mechanisms:

Chemo-topotaxis implies that a cell "is oriented in line with the source and moves toward or away from it" (Hand and Davenport, 1970). Such movements have been

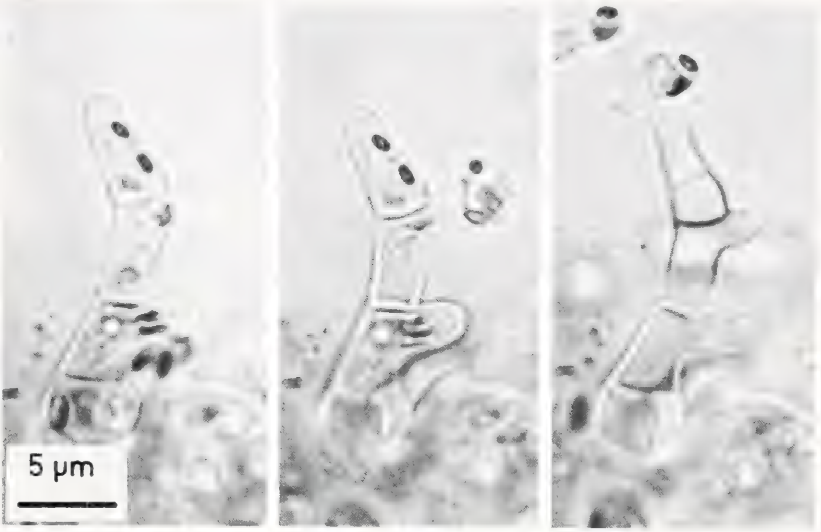


FIGURE 6. Spermatozoid release in *Laminaria digitata.*

demonstrated in the fern *Pteridium* (Brokaw, 1973) and in spermatozoa of the hydroids *Tubularia* and *Campanularia* (Miller, 1966, 1979; Miller and Brokaw, 1969).

Chemo-phobotaxis is defined as a re-orientation of the cell after crossing a critical

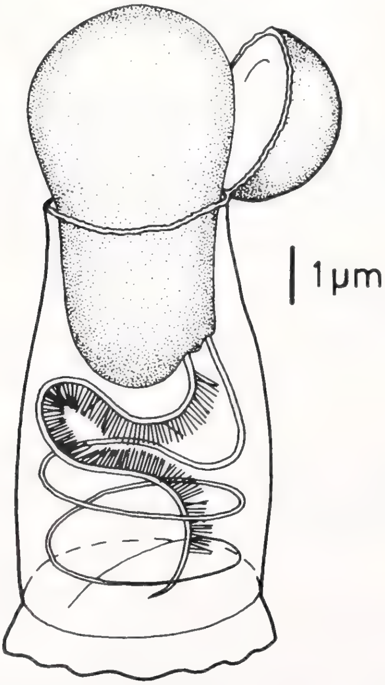


FIGURE 7. Schematic representation of spermatozoid release in *Laminaria digitata.* From Maier (1982).

concentration level (Ziegler, 1962). If this shock reaction occurs at decreasing concentration, the cell will eventually reach the source by "trial and error." Since the resulting accumulation of cells at the attractant source is identical in both cases, detailed analysis of the swimming behavior of individual cells is necessary.

Kineses are defined as modifications in locomotory activity due to variations in intensity of stimulation. These modifications refer to changes in linear velocity (orthokinesis) or to change of direction or angular velocity (klinokinesis, Hand and Davenport, 1970).

In Phaeophyceae, analysis of the locomotory pattern of male gametes in the vicinity of attractant sources indicates that possibly all three types of responses discussed above are represented.

III.5.1.1. *Chemo-thigmo-klinokinesis in* Ectocarpus

In *Ectocarpus siliculosus* analysis of video-recordings and cinemicrographic sequences (Müller, 1978; Geller and Müller, 1981) showed the following results: Male gametes swimming freely in seawater move in straight or slightly curved tracks with maximum velocity. When encountering a solid substrate, they tend to keep contact and move along the surface with reduced speed. *Ectocarpus* gametes have a heterocontic flagellation with both flagella inserted laterally. They seem to expose one side of the cell to the substrate, and loop-like tracks result. When viewed in the microscope, cells in contact with the cover glass move in clockwise loops. The cells are pulled by meandering beats of the front flagellum. Occasional beats of the obliquely inserted hind flagellum impose a strong lateral bias, resulting in a characteristic U-turn.

Presence of the attractant has two effects:

—The thigmotactic response of the male gametes is increased and their speed reduced.
—The beating frequency of the hind flagellum is increased proportional to the attractant concentration.

The combination of these two responses results in an indirect but very efficient "homing" effect:

—The thigmotactic response keeps the male gametes on the surface of the substrate where the settled female gametes are to be expected.
—The closer the male gametes get to the attractant source, the more frequent their hind flagella operate. This results in repeated U-turns or narrow circular paths around the female gamete (Fig. 8).

These behavioral changes of male gametes in the presence of ectocarpene can be described as chemo-thigmoklinokinesis, which means that the chemical stimulus modifies the locomotory pattern by increasing surface contact and frequency of angular impulses.

Upon arriving at the female gamete, the male gamete's front flagellum anchors to the membrane of the female gamete. This interaction is highly specific, and fails in inter-specific crosses (Müller and Gassmann, 1980) or even in non-compatible syngens of *Ectocarpus siliculosus* (Müller, 1979).

III.5.1.2. *Chemo-phobotaxis in* Fucus

In *Fucus spiralis* a crude analysis of spermatozoid movement in the vicinity of artificial attractant sources reveals that cells heading away from the source execute a characteristic U-turn. This reaction seems to take place at a specific distance from the

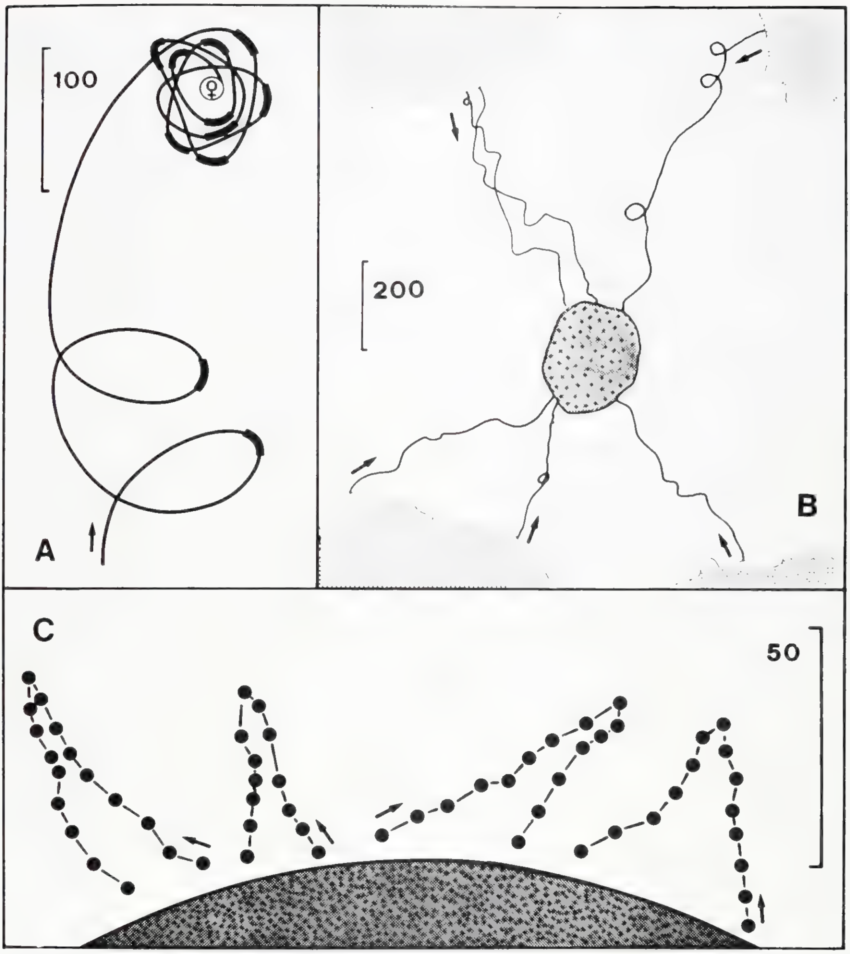


FIGURE 8. Tracks of male gametes approaching female gametes or artificial attractant sources. A: A male gamete of *Ectocarpus siliculosus* in contact with cover-slip (below), and in vicinity of female gamete (above). Broad sections of tracks indicate phases with bent hind flagellum. From Geller and Müller (1981). B: Tracks of several freshly released spermatozoids from 4 male plants of *Laminaria digitata* (corners) approaching a spherosil particle containing lamoxirene (center). From Maier (1984). C: Tracks of *Fucus spiralis* spermatozoids in the vicinity of a drop of FC-72 containing 10^{-5} molar fucoserratene. Time intervals between steps 0.1 s. Scale units in μm. Derived from video recordings.

source (Fig. 8). It indicates that passage through a critical concentration level induces a phobic reaction resulting in the cell's return to the source. In *Fucus* such movements occur in the free medium without contact with a solid substratum. Theoretically, this phobic reaction could account for trapping all spermatozoids that enter the egg's vicinity by random movement. However, a detailed analysis has not been carried out, and flagellar beat patterns are not known. The comparably long hind flagellum in *Fucus* (Manton and Clarke 1956; Fig. 9) as well as in *Ascophyllum, Pelvetia, Pelvetiopsis* and *Hesperophycus* (Henry, pers. comm.) creates hydrodynamic conditions that are significantly different from the situation in *Ectocarpus siliculosus.*

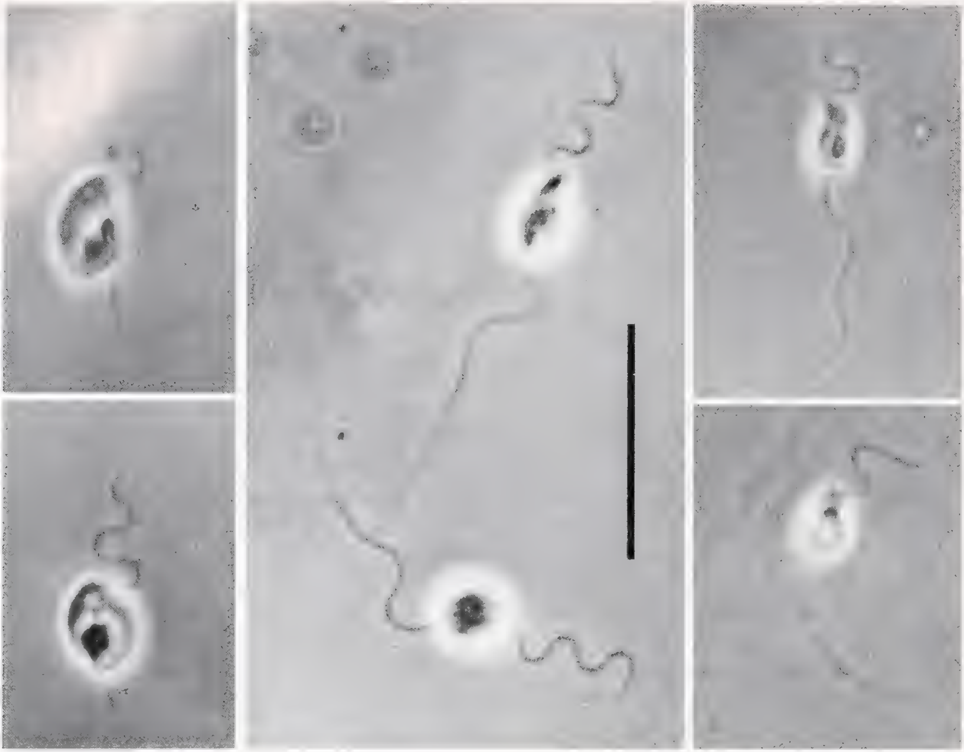


FIGURE 9. Male gametes of *Ectocarpus* (left), *Laminaria* (center), and *Fucus* (right). Scale bar 20 μm for all species.

III.5.1.3. *Chemotaxis in* Laminaria

Low-magnification video recordings of spermatozoid movement in *Laminaria digitata* indicate that the attractant source is approached rather directly over distances of at least 1 mm (Figs. 8, 10; Maier, 1984). Such a response requires the cell to probe the chemical gradient and orient its axis correspondingly. It seems possible that the extremely long hind flagellum in *Laminaria* (Fig. 9) acts as a sensor for the chemical gradient along its axis. As in *Fucus,* however, details of flagellar beat patterns are unknown.

III.5.2. *Bioassay and threshold concentrations*

A quantitative assay for gamete chemotaxis has been developed (Müller, 1976; Fig. 11) to examine the specificity of pheromone recognition systems and to determine the effective concentration ranges of specific pheromones and synthetic analogues. This method allows one to confront male gametes with known pheromone concentrations under controlled and reproducible conditions.

For chemotaxis experiments dilution series of synthetic pheromones are prepared in inert, water-insoluble fluorinated hydrocarbons with high specific gravity (FC-78, FC-72, supplied by 3M Company, Düsseldorf). Synchronous release of male gametes from gametangia in many species can be obtained by natural stimuli such as exposure to light after a dark phase. In many species, gametes can be concentrated by phototaxis.

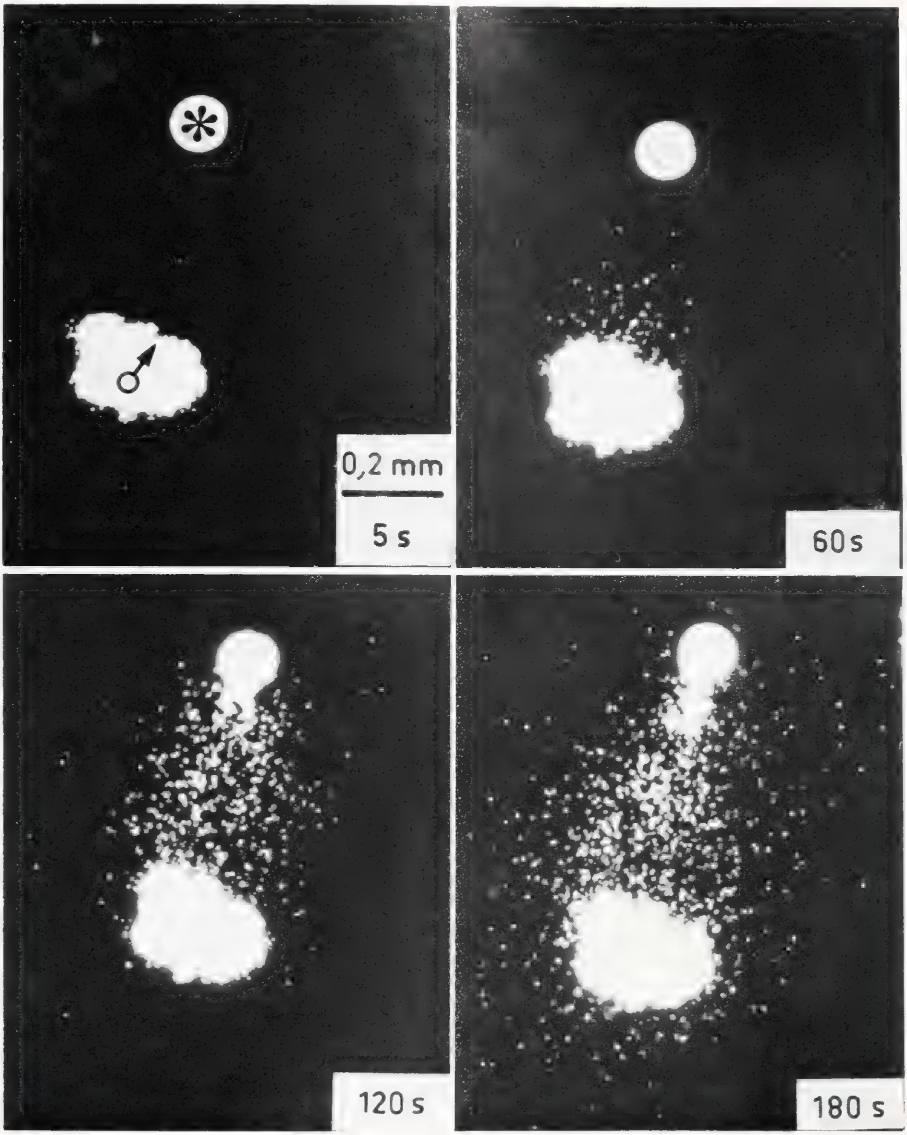


FIGURE 10. Spermatozoid release and subsequent chemotaxis in *Laminaria digitata* caused by addition of a "Spherosil" particle impregnated with lamoxirene. Dark field, microflash exposure. From Maier (1982).

In some Fucales, receptacles of male plants are treated with fresh water and kept emersed at +4° to 10°C. Sperm release is induced by subsequent immersion in sea water. In Laminariales, immediate sperm discharge can be induced by temperature shock.

For each experiment, a stainless steel ring (inner diameter 12 mm, 1 mm high) is fixed with a small amount of vaseline to the bottom of a polystyrene petri dish. The ring is filled with culture medium. Three 0.1 μl droplets of a pheromone solution and one reference droplet containing pure solvent are placed in the center of the well with

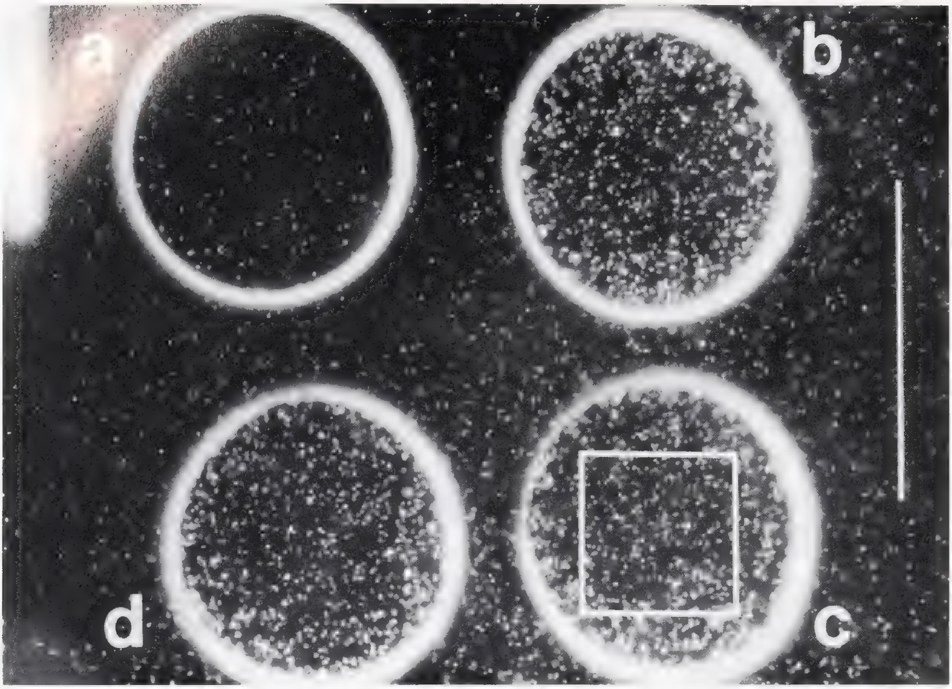


FIGURE 11. Quantitative bio-assay with spermatozoids of *Cutleria multifida*. Experimental droplets (b, c, d) contain 3.1 × 10^{-5} molar multifidene in FC-78. Blank droplet (a) is pure solvent FC-78. Scale bar 1 mm. From Müller (1976).

a microliter syringe. Male gametes are added and dispersed uniformly. When working with phototactic gametes, the preparation is protected from unilateral light and the microscope illumination is switched off. After a fixed time, normally 4 minutes, the distribution of the gametes in the preparation is documented by a flashlight microphotograph.

The assay is based on counting cell numbers within a standard area on pheromone-containing droplets (Fig. 11) and comparison with the cell density on the control droplet in repetitive experiments, followed by statistical treatment of the data. The concentration of pheromones refers to the aqueous phase immediately adjacent to the surface of the droplets and is determined by using the partition coefficient characteristic for the system (Boland *et al.*, 1981). The threshold concentration is defined as the pheromone concentration, that results in the lowest statistically significant attraction coefficient "Q-value" for the experimental droplets. Variability due to biological and technical parameters is determined in blank experiments with four solvent droplets. In brown algae, thresholds for chemotaxis towards the species-specific pheromones are usually in the concentration range of 10^{-11} to 10^{-9} *M* (Müller, 1976; Müller and Seferiadis, 1977; Boland *et al.*, 1981; Müller *et al.*, 1982b, 1984, 1985b;). The lowest threshold concentration ever measured was 6.1 × 10^{-13} *M* for chemotaxis in *Scytosiphon lomentaria* (Müller *et al.*, 1985). The sensitivity of the recognition systems is remarkably high. The authors concluded that even perception of single pheromone molecules can cause a gamete response (Boland *et al.*, 1984). With the pheromone secretion rates observed (Chapter III.3.), the spatial range of pheromone systems extends up to a radius of some millimeters.

III.6. *Pheromone-receptor interactions and specificity*

In many cases, female gametes secrete different compounds simultaneously (III.2, III.3). Generally the bouquet components are of similar chemical structure and possess similar physical properties such as solubility, dipole moments and electron polarizability. However, in each case only one compound acts as the potent specific pheromone. This is not necessarily related to concentration ratios. Presumably the pheromone molecules bind to specific receptor sites which are probably located in the male gamete's cytoplasmatic membrane.

To examine specificity and discrimination in ligand-receptor interaction, comparative activity tests were made with *Cutleria multifida*. These employed specifically designed synthetic multifidene-analogues in quantitative chemotaxis assays (III.5.2; Boland *et al.*, 1981). The concentration/response curves (Fig. 12) are similar for all attractants applied, but except for halogenated derivatives no substituent for multifidene, the natural messenger, reached a comparably low threshold concentration (6.5 × 10^{-12} *M*). It was concluded that the same receptor binding sites were responsible for binding all compounds, but that the stoichiometry of the pheromone-receptor interaction is unknown. Comparison of the chemotactic response to multifidene with that to derivatives with elongated side-chains, additional alkyl-substituents, or trans-isomers, showed that an accurate fitting of the pheromone molecule into the postulated receptor site is essential for biological activity. The activity-reducing effect of methyl-substituents in different positions is not identical, and indicates several different areas at the binding site which may allow turning of hindering substituents out of the area of contact. Partial hydrogenation of the signal molecule dramatically increases the

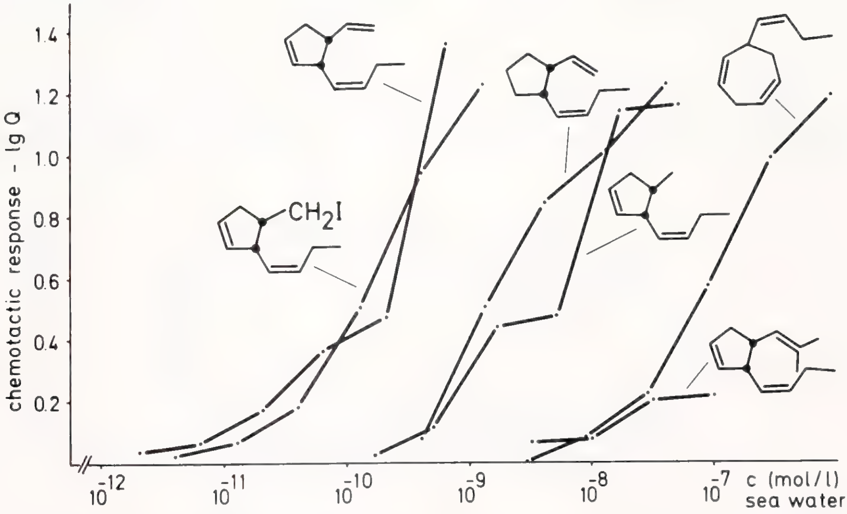


FIGURE 12. Concentration/response curves for chemotaxis in *Cutleria multifida* with multifidene and selected analogous synthetic compounds. After data from Boland *et al.* (1981, 1982b).

threshold in a non-linear relationship, indicating cooperativity of double bonds in binding.

From these results, a functional receptor model was developed (Boland *et al.*, 1981). Precisely fixed electrophilic groups complementary to the position of double bonds of the pheromone molecule are postulated at the receptor site (Fig. 13). Binding might be effected by concerted dipole-induced polarization of the ligands' π-electron systems. Each double bond in itself and the exact spatial arrangement one to another are important in such weak van der Waals-interactions, where binding energy decreases with distance by $1/r^6$. Thus, brown algal pheromones carry their "information" in form of electron orbitals in spatially fixed positions. Cyclic structures restrict the number of possible conformations. The receptor surface must almost exactly mirror the pheromone structure. Hydrophobicity in an aqueous environment might facilitate pheromone recognition and result in a high signal-to-noise ratio (Boland *et al.*, 1982b). Hydrophobic interactions and exclusion of water might also be involved in trapping and binding by increasing binding energy. Synergistic effects with constituents of pheromone bouquets have not yet been observed.

Substitution of the essential vinyl- by a iodomethyl- or bromomethyl-group with nearly identical van der Waals' radius reconstituted biological activity, thus supporting the tentative binding model. Substituents of comparable size and polarizability, inclusive of those with small dipole moments, may simulate the binding properties of the original vinyl-group. In a homologous series of halogenated derivatives higher activities could be directly correlated with increased polarizability and electron-donor properties (Boland *et al.*, 1982b).

The concept implies strict chiral discrimination of enantiomers. In fact, chiral recognition of pheromones has now been demonstrated in *Cutleria multifida, Ectocarpus siliculosus, Syringoderma phinneyi, Desmarestia aculeata, Chorda tomentosa, Sphacelaria rigidula, Xiphophora chondrophylla,* and *Adenocystis utricularis* (Boland *et al.*, 1984a, 1985; Maier *et al.*, 1984; Müller *et al.*, 1985a, b). The principles of the binding model developed with *Cutleria multifida* have been successfully applied to these species. In contrast, sperm of *Durvillaea potatorum* did not distinguish between the enantiomers of hormosirene (Müller *et al.*, 1985b).

III.7. *Interspecific cross-reactions*

Among the known brown algal pheromones (Table II) the possible number of modifications is obviously limited, due to restrictions in biosynthesis and the necessarily

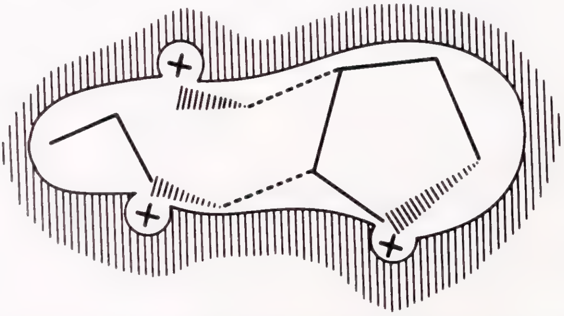


FIGURE 13. Proposed binding site of multifidene, showing polarization of the ligand's double bonds by complementary electrophilic groups at the receptor molecule. From Boland *et al.* (1981), modified to show the 3S,4S-conformation of (+)-multifidene.

conservative character of pheromone systems. Thus, complete species-specificity cannot be expected. This is confirmed by the finding of an order-specificity for lamoxirene in Laminariales, and the presence of multifidene in at least two species from divergent phylogenetic lines, *Cutleria multifida* and *Chorda tomentosa.* In many cases, female gametes produce considerable amounts of by-products in addition to their specific pheromones. These may act as male-attractants in other species. Moreover, the signal compounds are similar in respect to their physico-chemical properties in the reception process (III.6.), particularly in their overall π-electron distribution. For example, ectocarpene and multifidene show nearly identical topology in an appropriate conformation, including double bond arrangement (Fig. 14). The basic molecular mechanism of pheromone-receptor interaction can be assumed to be identical in all brown algae (Boland *et al.,* 1983b; Boland *et al.,* 1984a; Müller *et al.,* 1985a).

In high-sensitivity recognition processes with the potential for one-molecule effects in extreme cases, as seen in certain brown algal pheromone systems (Boland *et al.,* 1984a), interspecific interferences can be expected. Under laboratory conditions, interspecific cross reactions are easily demonstrated. Because of the conformational resemblance of ectocarpene and multifidene (Fig. 14), *Cutleria* male gametes react toward ectocarpene in the same manner as to their own pheromone, multifidene (Müller, 1976; Boland *et al.,* 1983b), although at approximately 400-fold higher concentration. This difference can be attributed to the loss of one coordination point caused by the shifted position of one double bond (Boland *et al.,* 1981, 1983b). Conversely, male gametes of *Ectocarpus* are attracted by *Cutleria* female gametes and to eggs of *Laminaria* and *Alaria,* which secrete ectocarpene as a component of their pheromone bouquet (III.2.). Desmarestene, the pheromone of *Desmarestia,* and related compounds inhibit chemotaxis stimulated by ectocarpene in *Ectocarpus siliculosus* (Boland *et al.,* 1984a). Interspecific interactions in Laminariales (Maier, 1984) and Fucales (Müller and Seferiadis, 1977; Fig. 15) result from common pheromone systems. Cross fertilization is prevented by species specific surface components involved in gamete recognition (Bolwell *et al.,* 1979). Common or related pheromones, spatial proximity, and temporal coincidence of reproduction are absolute prerequisites for such cross reactions. This problem of interference is especially evident for organisms that rely exclusively on sexual reproduction for propagation. To some extent, species of Laminariales avoid pheromonal interference by shifting their time of egg release and fertilization to the night (Lüning, 1981; Maier, 1984).

Production of pheromones or related compounds in vegetative thalli such as in

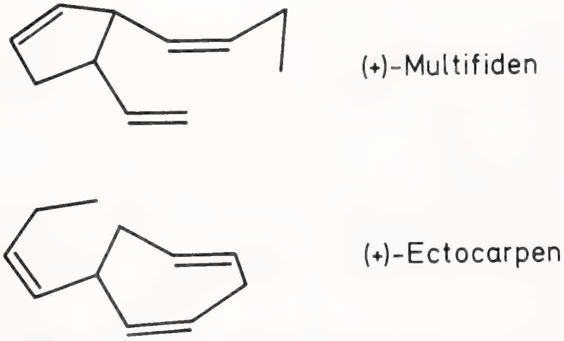


FIGURE 14. Topological similarities of the two pheromones ectocarpene and multifidene.

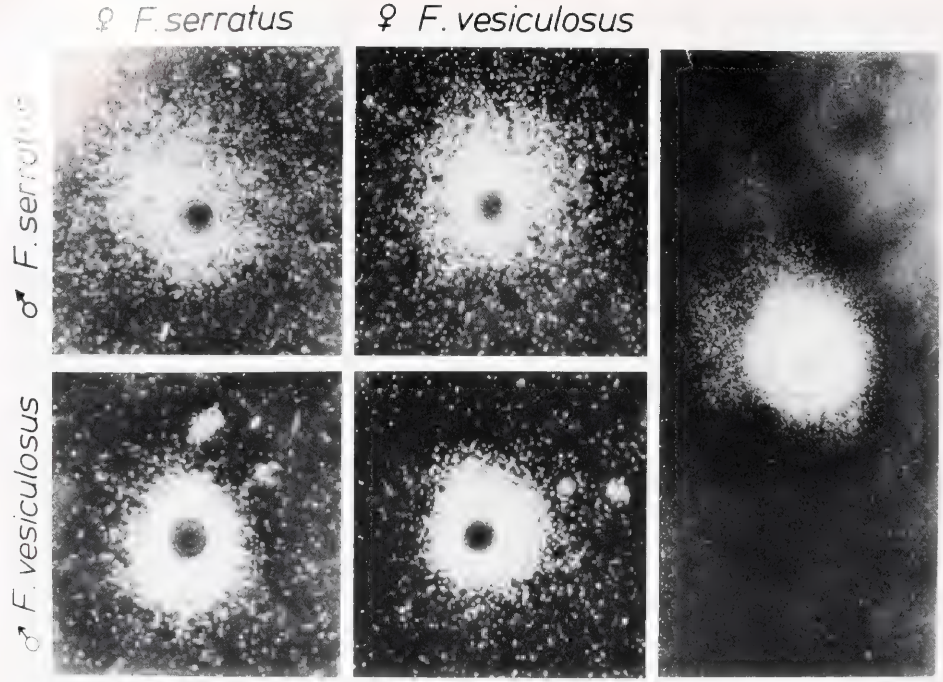


FIGURE 15. Cross-attraction between eggs and spermatozoids in two species of *Fucus*. Artificial pheromone source (right) contains 10^{-4} molar fucoserratene in FC-78. From Müller and Seferiadis (1977).

species of *Dictyopteris* (Moore, 1977) may function in competition for space or control of epiphytes. The significance of such interspecific interactions in nature is unknown and experimental approaches are lacking. Different strategies for successful "chemical competition" have been discussed by Boland *et al.* (1983b).

III.8. *Evolution of pheromone systems and chemotaxonomy*

Biogenetically closely related pheromones acting as chemical messengers in sexual reproduction have been found over the whole taxonomic range of brown algae (Chapter III.2. Tables II, III). Therefore, this type of gamete communication must date back at least to the emergence of the class Phaeophyceae, perhaps 600 million years ago in the Precambrian (Schopf, 1970). In view of this enormous geological time-span, the observed modifications in pheromone structure and receptor properties are extraordinarily small. This very conservative character reflects the fundamental significance of sexual reproduction in general and pheromonal interactions in particular in life histories and phylogeny of brown algae.

Occurrence and specificity of sexual pheromones have been examined in great detail within the order Laminariales (Maier, 1984; Müller and Maier, 1985). The activity of lamoxirene on spermatozoid release and chemotaxis in three families reveals evolutionary stability of pheromone specificity in these taxa in at least 10–20 million years since splitting of the order (Maier, 1984). On the other hand, the Phyllariaceae and Chordaceae (especially *Chorda tomentosa*) are clearly demarcated, and this is supported by morphological studies (Maier, 1984). Obviously, sexual pheromone systems have some significance as chemotaxonomic markers on the family- or order-level.

It appears quite unlikely that a mechanism as complex as pheromone-triggered spermatozoid release should have evolved independently in at least three orders: Laminariales, Desmarestiales, and Sporochnales (III.4.). Striking similarities in additional aspects of sexual reproduction have been emphasized by Schreiber (1932) and Clayton (1984b). A phylogenetic affinity among spermatozoid-releasing pheromones is therefore implied. A relation between lamoxirene and desmarestene is probable, since exchange of the epoxide-configuration of lamoxirene (Table II) with a double bond at the same position maintains a similar polarizability (III.6.). In Sporochnales, studies on sexual pheromones are in progress. First results indicate that the formula of the *Perithalia* pheromone seems to be identical with that of lamoxirene ($C_{11}H_{14}O$; Müller *et al.*, 1985c).

Use of (+)-multifidene as messenger in the two species, *Cutleria multifida* and *Chorda tomentosa*, is probably a convergent development. The topological resemblance of multifidene and ectocarpene (III.6.) might point out a possible way to the understanding of evolutionary processes in brown algal pheromone systems. The response of *Cutleria* male gametes to (+)-ectocarpene was mentioned above (Chapter III.6). In *Chorda tomentosa*, (+)-ectocarpene acts as a potent antagonist in spermatozoid release. For example, (+)-ectocarpene at a concentration of 3×10^{-10} *M* raises the threshold for (+)-multifidene 100-fold to 1×10^{-10} *M* (Maier, unpub.). Thus, the concentration of ectocarpene in egg secretions of *Chorda tomentosa* is high enough (Maier *et al.*, 1984) to interfere with its own recognition process. As a competitor, (+)-ectocarpene shows an even higher affinity for the receptor than (+)-multifidene, the genuine pheromone. Multifidene and ectocarpene are produced by the same biosynthetic pathway from a common precursor (III.3.). Thus, an evolutionary link between multifidene- and ectocarpene systems becomes highly likely. Ectocarpene has been proposed as the most ancient pheromone (Müller *et al.*, 1985a). Alternating convergent differentiation of chemoreceptor and pheromone biosynthesis must be assumed, but it is not possible to decide which one changed first and why, or whether a genetic coupling can be excluded.

Differentiation of the ectocarpene receptor has been studied in the distantly related species *Sphacelaria rigidula*, *Ectocarpus siliculosus*, and *Adenocystis utricularis*. Different responses to variations in the butyl side chain of ectocarpene in quantitative chemotaxis tests (III.5.2.) revealed individual modifications of receptor topology and charge distribution (Müller *et al.*, 1985a).

IV. Concluding Remarks

The findings summarized in this review illustrate convincingly that aquatic habitats not only provide space and a suitable physicochemical environment for algae, but also that water serves as a carrier for a variety of chemical signals that coordinate among other things reproductive activities of organisms. We should be aware that the systems discussed here are just a first glimpse at what occurs in chemical communication. It can be assumed safely that other groups of organisms, including animals up to the vertebrate level, use chemical messages for various purposes. The detailed analysis of brown algal pheromones was facilitated decisively by the volatile nature of the compounds involved. Due to methodical limitations even in this case, our knowledge is possibly confined to only one facet of the reality.

At the present state, a possible function of primarily intraspecific pheromones in relations between different species is only a matter of speculation. Aquatic ecosystems also contain numerous non-specific substances excreted by organisms as waste products and from decomposition processes. Pheromone systems functioning in such a complex background must be remarkably specific. The effects of anthropogenic pollution on such systems cannot be evaluated competently at present for lack of detailed data. There are very few experimental findings that do indicate anthropogenic interference with pheromone systems in aquatic habitats. A study by Steele (1977) on the effect of fossil oil pollution on fertilization of *Fucus edentatus* shows that such effects are possible.

ACKNOWLEDGMENTS

We express our thanks for critical discussions and valuable comments to W. Boland, M. N. Clayton, E. C. Henry, L. Jaenicke, and A. Peters.

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FLASH CONTROL AND FEMALE DIALOG REPERTORY IN THE FIREFLY *PHOTINUS GREENI*

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ABSTRACT

In the *Photinus greeni* courtship dialog the male emits flashes in pairs (S1S2) about 1250 ms apart at 25°, the pairs recurring every 5 to 7 s. The female answers with one flash, usually about 750 ms after the male's S2 signal (S2R response). S2R latency includes 400+ ms of central nervous delay. Using paired signals of electric light, female responsivity to stimulation at different frequencies was established and excitatory state modified so that S1Rs (responses to the first flash of signal pairs), and spontaneous flashing, became more frequent. S1Rs have a longer latency than S2Rs.

Flash timing was examined for presumed neural noise, statistical and individual variation, persistence, response cycling, hyperexcitation, fatigue, and habituation. A model central neural flash-control mechanism, based on an excitability transient rising from a resting level to a flash-triggering level, distinguishes S1R from S2R and accounts for much behavioral timing.

The female clearly has an *input*-timing element, used normally for identifying the male's signal pair. Since females sometimes emit pairs of spontaneous flashes at about the same average interflash interval as the male's, it is suggested that her timer may, under stress, assume the *output*-timing role normal in the male.

INTRODUCTION

Because of the unique time-coded flashing in the sex-recognition dialogs of many lampyrid fireflies and the ease with which their signals can be simulated, several courtship protocols have been studied intensively. Field investigations have revealed species-specificity and great diversity in coding, while signal simulation has made it possible to quantify visual, timing, and response parameters and relate them to rhythm generation and other processes in the firefly central nervous system.

In *Photinus greeni* (Lloyd, 1969) the flying male's periodic advertising luminescence is a pair of flashes, or "phrase" (Lloyd, 1966), rather than a single flash. The stationary female times her flash from the second signal (S2) of properly timed flash pairs (S1S2). The dialog is a particularly attractive study system because both intra- and interphrase timing can be varied experimentally.

Buck and Buck (1972) reported that at 27°C the flashes in the male's phrase had a duration of 100 ms and were about 1300 ms apart. Phrases were repeated about every 5 s while the male was flying and, less regularly, after he had landed and was walking toward the female. The female answered with a 200 ms flash after the male's second flash. The average response latency to simulated male phrases was about 850 ms. Like the females of many other *Photinus* fireflies, the *P. greeni* female does not require successive male signals to be presented rhythmically, tolerates much variation

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Received 30 August 1985; accepted 19 January 1986.

Abbreviations: S1R, response to first flash of stimulus pair; S2R, response to second flash of stimulus pair; S1S2, interval between flashes of stimulus pair; SP, spontaneous flash.

in signal duration and intensity, and twists her abdomen when flashing, aiming her light toward the male.

Using manually controlled simulations of the male's stimulus phrase (S1S2), Buck and Buck (1972) showed that the acceptable intra-phrase interval could differ substantially from female to female and that the range was wider than that actually used by males in the field. They noted an occasional response by the female to the first flash of the male's phrase pair (S1R) but regarded such irregularities as rare. They also concluded that the female, after being stimulated by S1, is refractory to further photic input for about a second.

In several firefly species the delay between light reception by the eye and the occurrence of the response flash much exceeds the delay between direct electrical stimulation of eye or brain and the resulting flash (Case and Buck, 1963; Magni, 1967; Hanson *et al.* 1971; Buck *et al.*, 1981B). Only a few milliseconds could reasonably be allocated to visual processes (Case, 1984). The interval between arrival of the visual message in the brain and the departure of the motor neural message from the brain to the (abdominal) light organ thus constitutes a specific central nervous delay, which is evidently by-passed by electrical stimulation. In the *P. greeni* female the electrical latency is 250–300 ms (Case and Buck, in prep.) so at least 400 ms of the photic latency (range 650–950 ms) must be central delay.

It is very helpful, in experimenting with photic response, that (1) the nearly hemispherical eye is very sensitive to light normal to any part of the corneal surface, (2) both sexes are extremely tolerant of differences in signal flash intensity (as expected from the fact that they court over a distance range of several meters; Buck and Buck, 1972: Case and Buck, 1973), and (3) firefly dialog exchanges tend to be all-or-none: the individual either responds fully to the signal or does nothing. In the *P. greeni* female the range of acceptable photic stimulus intensities is about 10^4 (Case and Buck, in prep.). Unrestrained specimens can thus be used in behavioral work with good assurance that all signals of moderate intensity will be seen except those from directly behind.

In the present laboratory investigation we recorded females' dialogs with artificial signals of controlled intensity, duration, number, and timing and analyzed response latency and sequence in relation to phrase repetition frequency and pattern. We also studied spontaneous flashing and the usually infrequent and seemingly anomalous response to the first flash of the stimulus pair (S1R). A main objective was to explore timed elements in flash control and photic dialog in intact, unrestricted females as indications of neural circuitry involved.

The species-specificity of firefly dialog led historically to the impression that each photic code is rigid and invariant. *Photinus macdermotti* shares with *P. greeni* a code involving paired male flash signals and a singly flashed female reply. In this species, differences between signal timing by answered and unanswered males and other variable behaviors have been reported (Lloyd, 1969, 1981, 1984; Carlson *et al.*, 1976, 1977). Such observations appear to widen the potentialities of firefly communication systems. Hence, in the present analysis of flash timing in *P. greeni* we paid particular attention to the lability of female photic behavior and to behaviors reported to exist in *P. macdermotti*.

MATERIALS AND METHODS

Females of *Photinus greeni* were collected between 8:30 and 11:00 p.m. EDT at several sites in Woods Hole, Massachusetts, during June, July, and August of six summers, mostly 1972–1974. Animals were located on perches in vegetation by their replies to paired flashlight signals. Specimens were stored in dim room light in 35 × 65 mm plastic vials with white snapcaps, humidified with paper toweling dampened with dilute sucrose solution, and used for up to 10 days. Prior to experimentation each female was dark-adapted for at least 15 minutes and tested repetitively with paired signals until response was stabilized. Experiments were run between 6:00 a.m. and 11:00 p.m.

Usually four females were tested simultaneously in a light-tight box 1 m long in which a female was stationed in each of four 7 × 10 × 15 cm compartments at one end, shielded from the other females by light baffles (Fig. 1). Controlled flashes from one or from two Sylvania 911 glow modulator lamps were conducted singly or consecutively by light guides to a diffusing surface at the other end of the box so as to illuminate each compartment equally. The response flashes of each female were detected separately via an RCA 1P21 photomultiplier viewing her chamber from above. Each female was placed in a chamber still in her residence vial, which was inverted so that the white cap would reflect her flashes to the photomultiplier. Durations and times of presentation of glow lamp flashes were controlled by a pair of Grass S44 stimulators.

Temperature in one vial and in the main chamber was monitored via separate thermistors. Temperatures during experimentation varied between 21° and 24°C at the start of a day's work and commonly rose about 1° by the end. In another study we found temperature coefficients of several elements of the female's photic response to be slightly above 2 (Case and Buck, in prep.). Ambient temperatures thus should be considered when measurements from different days are compared or pooled. However, most of our data are from single days (maximum 1° change) and often from

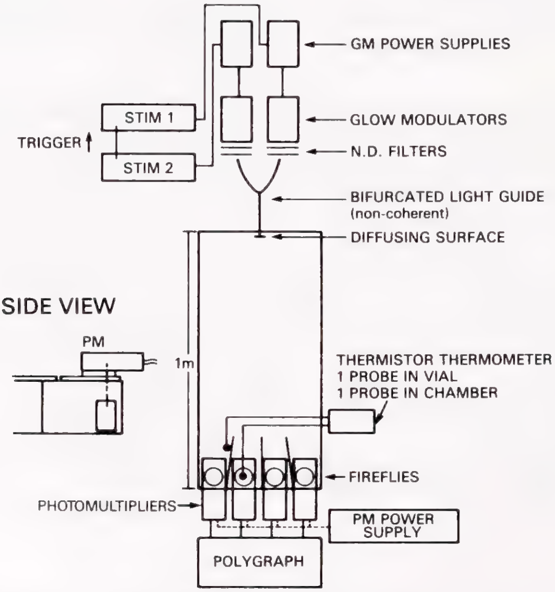

FIGURE 1. Apparatus for inducing and recording photic response. Flashes of glow modulator tube light, with duration, intensity and sequence controlled by physiological stimulators, were delivered to a diffusing surface viewed by 4 in-vial female fireflies, each in a separate chamber, at the other end of the box. Each firefly's light was detected from above by a separate photomultiplier photometer. Output from photometers was recorded on multichannel chart paper.

females in the same run. Also, as shown below, differences between response latencies of different females tested together, or even between means for the same individual at different times in the same experiment were sometimes highly significantly different. We have not normalized the data because we feel it would give a deceptive appearance of uniformity and obscure what we consider an important feature of firefly communication, the variability in timing.

Stimulus artifacts and responses of females were recorded on separate channels of a Grass Model 7C polygraph. Latencies were read to the nearest 5 to 100 ms, as appropriate to chart speeds from 100 mm to 5 mm/s. Timing was from rise point to rise point.

We did not measure flash intensities because of possible variations connected with abdominal aiming (Introduction) and because we often chose recording levels in which the detector was saturated at flash peak in order to get more accurate latencies.

Statistical variation is indicated as mean ± standard deviation (σ), not standard error, and by V, the coefficient of variation.

RESULTS

1. Warm-up behavior

In broadcasting simulated male phrases while collecting females we found that specimens in the field not uncommonly responded to some S1 signals, or even to both S1 and S2 successively. Typically also, females that had been in room light and were then subjected to darkness and exposed to rhythmic S1S2 pairs usually did not respond during the first few minutes and then began an irregular mixture of S1Rs and S2Rs, often interspersed with flashes that occurred 2 s or more after signals. Since data presented below (results, Section 7) showed that exogenously stimulated photic responses occurred only between 600 and 1800 ms post-stimulus, flashes with longer latencies were classified as spontaneous (SP, Fig. 2).

Before females responded regularly they usually required several minutes of stimulation at 20 to 30 s intervals. In 43 randomly chosen runs begun between 7:00 a.m. and 10:00 p.m., the stimulation time necessary to reach sustained S2R responsivity ranged from 5 to 16 minutes ($M = 9.9 \pm 2.9$). In specimens that had been in the laboratory for a day or so there were no marked differences between morning and afternoon experiments. However, even passage of time and repeated presentation of proper signals were not enough to insure response if the female was not receptive. It was noticed repeatedly during video photography (Case and Buck, in prep.) that a freely walking female ignored dialog signals until she had mounted a suitable perch, come to a complete halt, extended her head from beneath the pronotum, and spread her antennae.

2. Stable S2R response

Response-ready females stimulated once every 15 s or less frequently tended to give uniform S2Rs to each consecutive phrase throughout long series, whereas those stimulated every 10 s, or more frequently, often developed response irregularities.

In one typical test series with 4 females exposed simultaneously to one pair of stimulus flashes every 30 to 45 s, two animals missed only a single response each in 156 consecutive cycles and the other two only 19 and 36 (33 at the start), respectively. Flash timing was highly regular, latency V values commonly being as low as 1.5 for 10 consecutive cycles. Each individual appeared to maintain flash intensity within narrow ranges (Fig. 3). Control observations in very dim light showed that responding

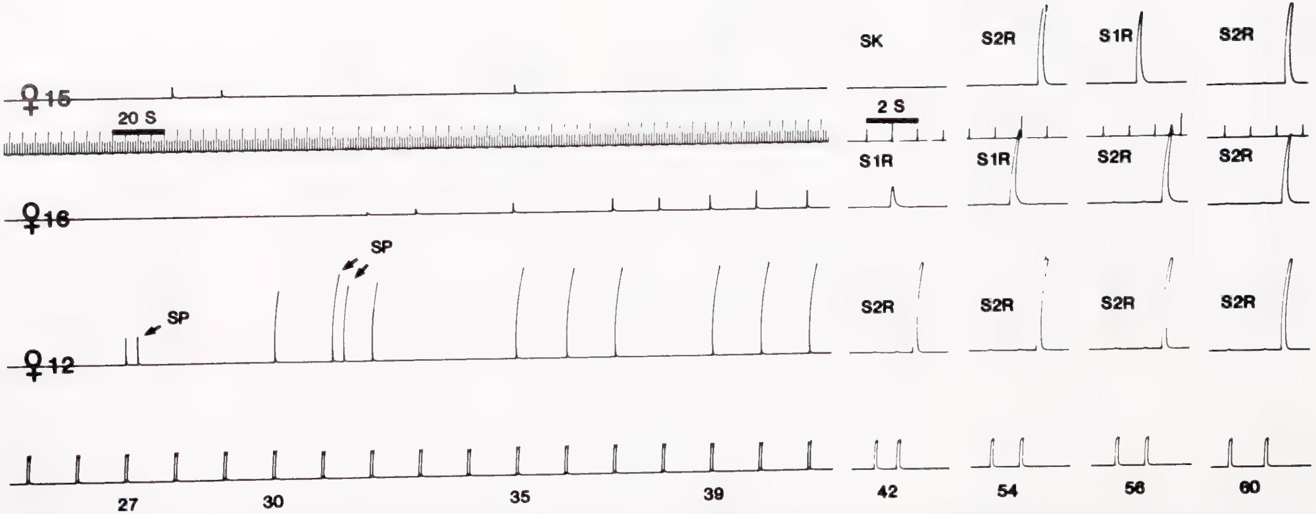

FIGURE 2. Typical flashing during warm-up. Three females were exposed to one phrase pair of 100 ms signals every 18 s (bottom channel). Female 12 (next to bottom channel) responded (S2R) first at cycle 27, 8+ minutes after setup, then to phrases 30, 32 and 35-7, with some interspersed spontaneous flashing (SP) also. At phrase 39, Female 12 began regular 1:1 response. Females 15 and 16 were slower in getting started. After phrase 41, chart speed was increased 10-fold to illustrate skipped responses (SK) and S1Rs still occurring in the Female 15 and 16 traces. All three animals were responding regularly by phrase 60.

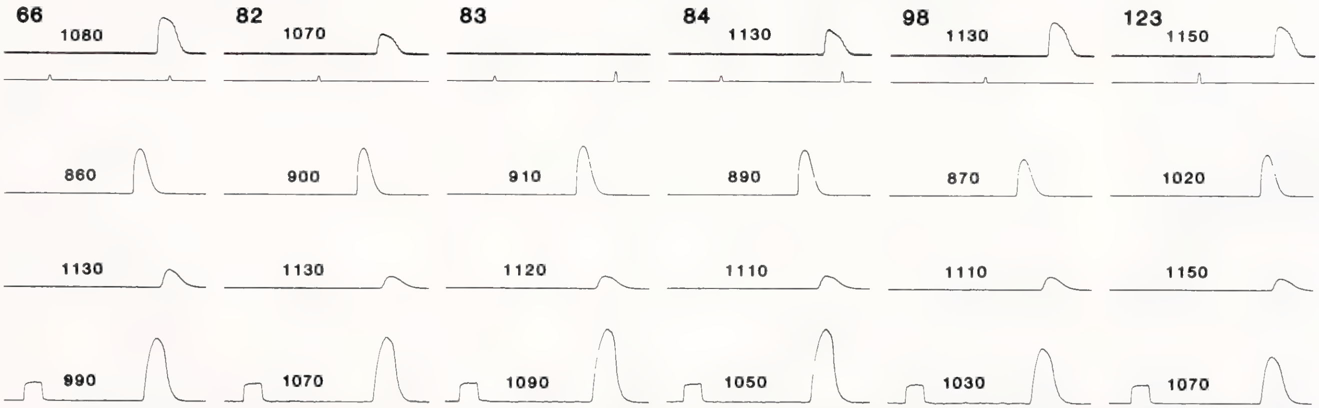

FIGURE 3. Responses of 4 females stimulated once every 30 to 45 s. Six cycles out of several hundreds are shown, with the S2 signal superimposed on the record of the bottom channel female. Response latencies in milliseconds are given to left of flash traces. Note individual constancy (horizontal sequences) and differences (vertical comparisons). Flash skipping was absolute (top female, episode 83). Paired 150 ms signals, 1500 ms apart; 21°; time marks (2d channel) 1 s.

captive females usually did not shift body position for long periods, so it is believed that the apparent uniformity in flash intensity was valid.

[illegible] other points of interest during slow driving were (1) that flash-skipping, if it occurred, was sudden and absolute, with intensity and latency of the first post-skip flash close to those of the flash just preceding the skip (Fig. 3, cycles 82–84, top channel; *cf.* also Figs. 5 and 6), and (2) there were marked and consistent inter-individual differences in latency. In the two most responsive females of the Figure 3 run the mean latencies (S2R) for 100 responses (21°) were 886 ± 27 ms and 1130 ± 38 ms, with Vs of 3, and highly significantly different from each other.

3. *Timing latitude in dialog*

Figure 4 shows the mean percentages of S2R responses for 13 females presented with 2900 S1S2 stimulus phrases ranging in duration from 550 to 1850 ms. Each phrase was presented two to four times at intervals of 10 to 15 s, followed by another group of phrases of different duration, and so on, some of the females being run again on a different day (total responses 1600). The heavy line shows that intra-phrase durations between 900 and 1400 ms evoked responses more than 50% of the time on the average. The horizontal lines show the ranges of essentially 100% response in the 11 runs evoking more than 70 responses each, with the dashed extensions indicating the shortest and longest phrases to which the particular individual ever responded. As with females stimulated at long intervals (Fig. 3), whenever an animal failed to respond, whether in the high response range or where response was rare, failure was not preceded by dimmer flashes but was sudden and complete as if the flash-triggering process were all-or-none.

Females exposed simultaneously to the same stimulus regimen often exhibited a remarkable degree of individuality. For example, in Table I, which lists the mean response latencies of 3 females during several 12-cycle sequences in a 200-cycle run,

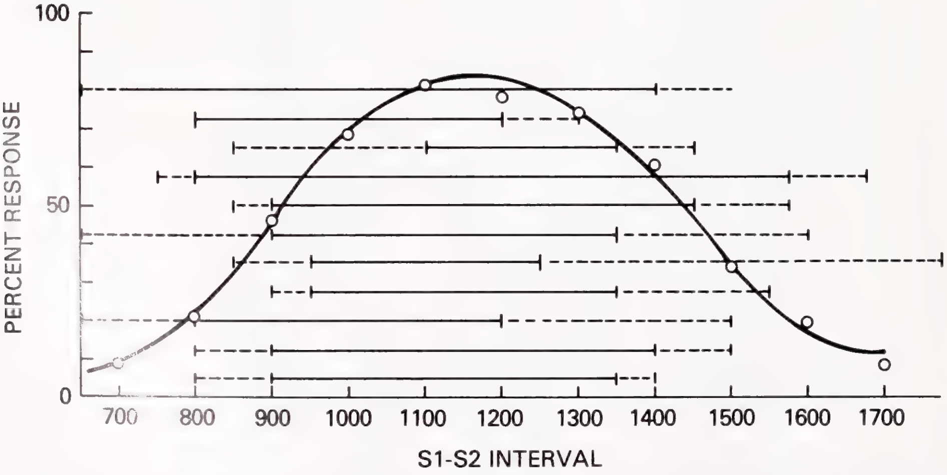

FIGURE 4. S2R percentage in relation to stimulus phrase duration (S1S2). Phrase interflashes between 900 and 1400 ms elicited 50% or higher responses per test (heavy curve). Horizontal lines are the nearly 100% response ranges for 11 runs with more than 70 responses each. Dashed extensions indicate exceptional long and short phrases answered. Thirteen females, 25 runs, 2900 phrases, 1600 responses, stimulus durations 100 ms, 23–25°, not normalized.

TABLE I

Mean response latencies of three females during a run of 200 successive S1S2 stimulus pairs of glow-modulator lamp flashes (25°)

Sequence	Cycles	Phrase presentation order	Female 4	Female 11	Female 14	t (signif.)
1	7–20	970, 1020, 1120, 1220, 1340	751 ± 46	—[1]	663 ± 30	4/14[2]
2	34–45	970, 1150, 1210, 1350	716 ± 71	629 ± 70	641 ± 36	4/11, 14
3	64–77	890, 950, 1040, 1140, 1220	751 ± 56	663 ± 31	628 ± 25	4/11, 14; 11/14
4	117–127	960, 1060, 1140	756 ± 35	645 ± 23	635 ± 37	4/11
5	128–139	1140	735 ± 33	665 ± 18	620 ± 8	4/11; 11/14
6	162–174	850	—[3]	752 ± 54	656 ± 18	11/14
7	185–201[4]	1140	766 ± 55	691 ± 56	653 ± 33	4/11, 14; 11/14
Means[5]			746 49	674 42	642 27	
t				6/2–7[6]	1/3,5	

[1] Female 11 did not begin responding until after the 20th phrase presentation.

[2] "4/14" means that the mean response latency of female 4 differed significantly from that of female 14 by t test at the 5% level or lower.

[3] Female 4 failed to respond to phrases of 850 ms duration. All three females failed to respond to phrases of 750 ms.

[4] 10 ms pulse in this series instead of usual 100 ms.

[5] Periods and standard deviations averaged independently.

[6] "6/2–7" means that the latency in sequence 6 of female 11 differed significantly from runs 2, 3, 4, 5, and 7 of that female.

each individual's mean differed significantly from those of one or both of the other females in nearly every possible pairing (horizontal comparisons). In the within-female comparisons (columns) there was less variation. Both between-female and within-female comparisons seemed independent of whether S1S2 phrase duration was changed frequently (lines 1–4) or held constant (lines 5–7), and of whether the comparison was made early or late in the run. Variability *per se* also differed markedly among females, individual V values ranging from 1.3 (Female 14, sequence 5) to 10 or 11 (Females 4 and 11, sequence 2), with an overall mean of about 5.

No correlation was found between response latency and stimulus phrase duration. Table II gives latencies to all phrases shorter than 980 ms and longer than 1340 ms

TABLE II

Mean response latencies in relation to S1S2 stimulus phrase duration

		Phrases <980			Phrases >1340	
Run	Female	n	M & σ	t	n	M & σ
1	4	34	768 ± 63	0	11	767 ± 90
2	11	44	685 ± 60	1.85	12	644 ± 89
3	14	59	682 ± 50	1.38	9	706 ± 32
4	14	27	726 ± 76	1.83	12	683 ± 47
5	18	12	774 ± 57	2.95	12	702 ± 62
6	9	34	730 ± 50	0	30	724 ± 56
Totals/Means		210	728		86	704

300 responses selected from a total of about 1000 tests on 5 females.
Runs 1–3 from one day's experiment, 4 and 5 from another and 6 from a third.

for 5 females with similar response ranges. There was no significant difference between short and long in 5 of the 6 comparisons, though there were considerable differences between Female 14's runs on different days and large differences between different females in the same run (No. 4 *versus* Nos. 11 and 14 in the first day's run).

4. *Frequency effects during rhythmic stimulation*

Females stimulated every 10 s or oftener not only responded less consistently than when phrase repetition period was longer (Section 2) but did not respond *consecutively* to stimulus pairs presented more frequently than about once every 4 s. Figure 5A–C shows a continuous record of S2Rs emitted by two females exposed to, first, 6 signal pairs (5 cycles) at 9 s intervals (5A: 3 responses by Female 1, 5 by Female 2); next, 16 pairs at one per 4.5 s (5B: 5 responses by No. 1, 11 by No. 2); and, finally, 12 pairs at one per 3.7 s (5C: 3 responses by No. 1, 6 by No. 2). In 5C, note that Female 2 responded regularly to *every other* pair.

When phrase presentation frequency was increased still further, so that stimulus flashes succeeded each other in an even rhythm (5D), it was, of course, no longer possible to associate responses with particular stimulus flash pairs. Assuming that the standard paired signal format was nevertheless still operating, the responses of Figure 5D were as if every third flash was not seen and flashes 2, 5, 8, etc., functioned as S2s in evoking responses. Runs of up to 12 consecutive "every third" responses were recorded. In all these, flash succession was still limited to a minimum of about 4 s and the interval between last effective S2 and next effective S1 was never shorter than 2.5 s. Also, response flash intensity usually decreased progressively as if the system was being pushed too hard and refractoriness was building up. Thus, in longer series, in which response typically failed at irregular intervals for several cycles at a time, the first flashes after resumption were considerably brighter (Fig. 5D) as if some depressing influence had dissipated. One of the Figure 5D females responded *repetitively* only when driven at intervals of 1125 ms or longer (38 runs) but gave an *initial* S2R (only) in each of 4 runs in a 1070 ms rhythm and of 3 runs at 1000 ms.

In addition to the usually regular S2R responses to rhythmic signal series, there were rare instances of response after the first signal of a series (5E), after the third (5F), after both second and third (5G), and of doubled flashes with the two elements only 300 ms apart (5H).

At rhythmic photic signal presentations faster than one per second, females never responded. One period tested, 580 ms, was interesting because alternate flashes in that rhythm were within the acceptable phrase range (2 × 580 = 1160 ms). The response failure thus indicated that a flash intercalated between S1 and a properly timed S2 was inhibitory (*cf.*, Buck and Buck, 1972).

5. *Fluctuating excitability during prolonged driving*

Persistent repetitive driving sometimes evoked sporadic and much increased frequencies of the three ordinarily rare events seen in warmup behavior (Section 1): flash skipping, spontaneous flashing, and S1Rs. Flashing series rich in these sporadics sometimes alternated with intervals of very regular response. Figure 6A–F illustrates response heterogeneity during a short portion of a 140 minute run of rhythmic driving. The subject female emitted S1Rs (A), then a few conventional S2Rs (B, cycles 7–10), then primarily S1Rs again (C, D, cycles 20–38), then flashed spontaneously (E), then gave more S1Rs (F) and so on. It might be questioned whether even the rhythmic flashing was actually responsive, but the in-series latencies were clearly non-random (*e.g.*, M = 1200 ± 82 ms in D, cycles 30–36).

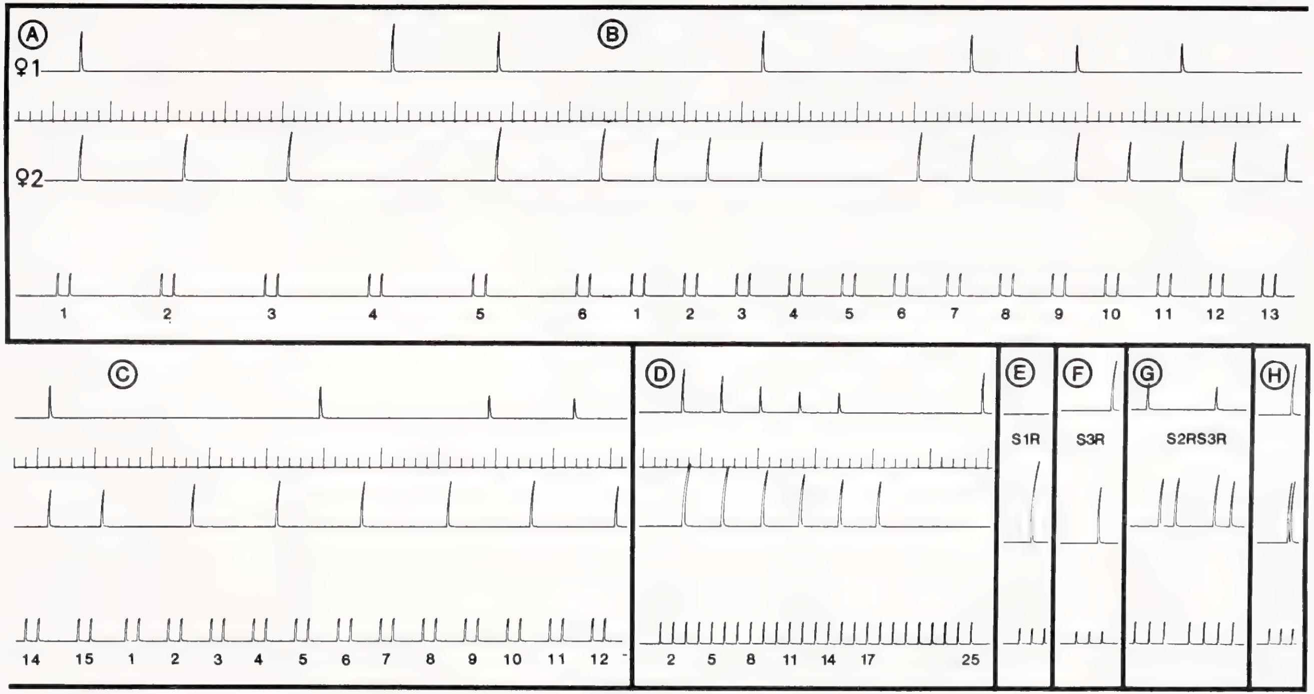

FIGURE 5. Responses of two females to rhythmic photic driving. Runs A through C continuous. A, 5 cycles at one phrase per 9 s; B, 15 cycles at one phrase per 4.5 s; C, 11 cycles at one phrase per 3.7 s. One second time comb (2d channel) applies to all figures. Except for 4 skips, Female 2 (3d channel) responded 1:1 at the 9 s and 4.5 s rhythms. At 1/3.7 s (C) she responded regularly to every other stimulus pair. Female 1 (top channel) responded only about half as often. D, responses to 1200 ms train were given after each third signal, with progressively decreasing intensity. All responses in A–D were S2Rs. Very rarely, a response occurred after S1 (E), after S3 (F), after each of two consecutive signals (G) or as a double flash (H). Different females in each series of D–H and different from those in A–C.

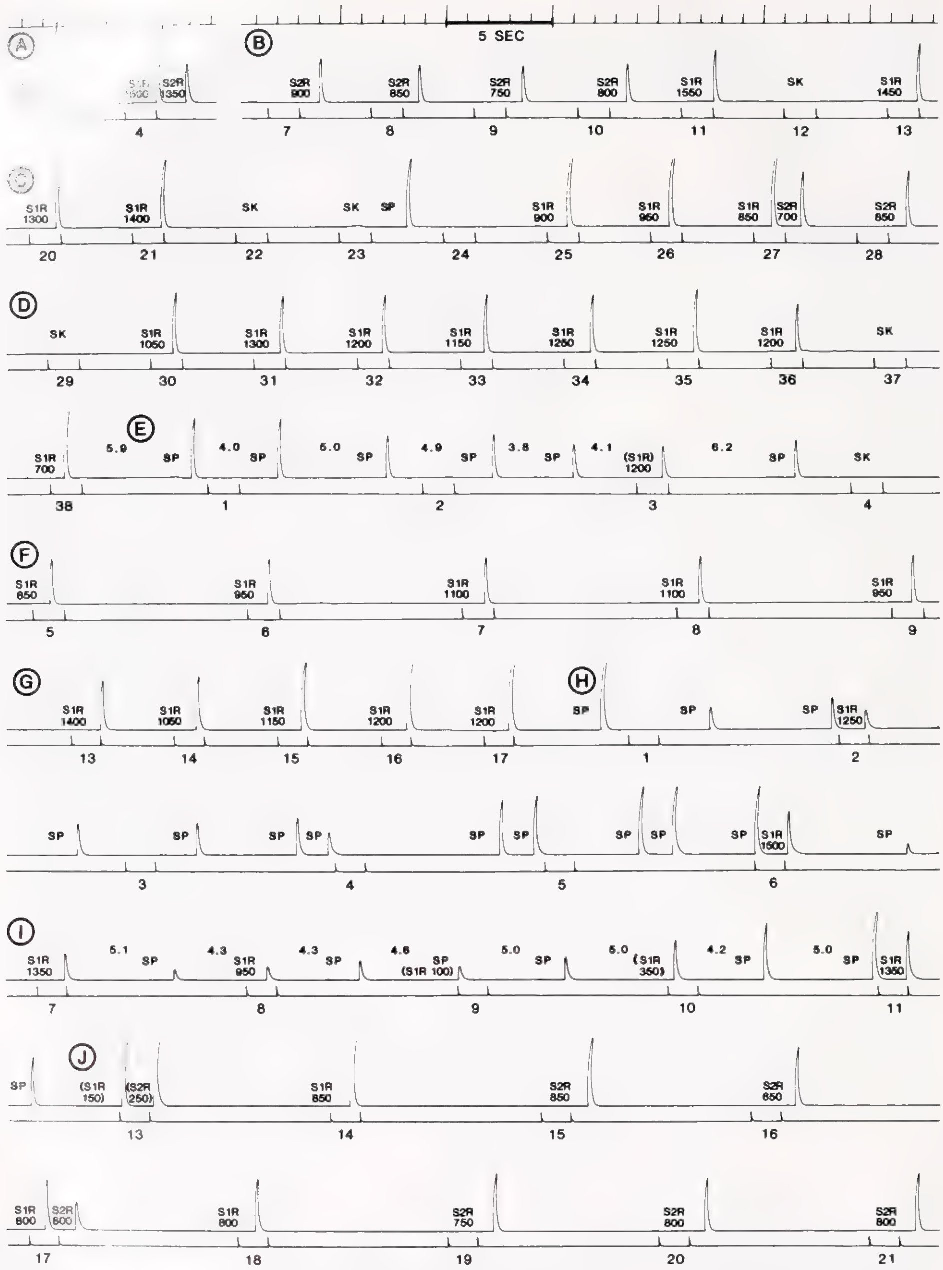

FIGURE 6. Fluctuating response during rhythmic photic driving. A–D, three sequences during 38 cycles of driving at one 1500 ms phrase each 5 s, showing alternating runs of S1Rs (phrases 3, 4, 11–27, 30–38), S2Rs (phrases 7–10, 27–28), skipped responses (phrases 12, 22, 37, etc.) and spontaneous flashes (SP). E–F, 9 cycles of driving at 1/10 s, during which there was first spontaneous flashing (1–4) then regular S1Rs (5–9). Except for indicated omissions, record A–F is continuous. G–J, continuous driving sequence from different part of record of same female, showing S1Rs at one phrase per 5 s (G), followed by single and paired spontaneous flashes (H) and a mixture of S1Rs and S2Rs during stimulation at 10 s intervals (J). Durations of some spontaneous interflashes given in seconds with decimals. Female 62; 22°; signal duration 2 ms. Latency values in parentheses interpreted as spurious or (E3) spontaneous.

We occasionally changed the driving frequency from one phrase per 5 s (A–D) to one per 10 s (E–F) but, as shown by a second sample series from the same run (G–J), there was no correlation between stimulation frequency and type of response. Not only did type of response not necessarily change with the signal, but the female showed herself able to maintain either regular 1:1 S1Rs or S2Rs at either 5 s or 10 s rhythms (*e.g.*, B, cycles 7–10 *vs.* J, cycles 17–21; D, cycles 30–36 *vs.* F, cycles 5–9). We attribute the fluctuating responses to the persistent stimulation. Insight into these behaviors, and implications for flash control mechanisms, were derived from further consideration of the relations between spontaneous flashing and S1Rs (Section 6–8, below, and Discussion).

6. *The S1R enigma*

S1R flashes were generally indistinguishable in intensity and kinetics from S2R flashes of the same animal, and the respective latencies were sometimes in the same range. A low-light video study, to be reported elsewhere, showed that captive females giving an S1R sometimes executed the lantern-aiming movement that almost invariably accompanies the normal S2R. Like spontaneous flashes, S1Rs were usually infrequent in comparison with S2Rs. In more than 9500 box tests during one season there were only 64 S1Rs and 30 instances in which females flashed after both S1 and S2 (S1RS2R) whereas there were several thousand S2Rs; and of 47 females tested only 23 gave any S1Rs. Such evidence suggests that an S1R is due to a central nervous timing perturbation that causes an S1 to be treated as an S2 (see further).

Though most females did not produce enough S1Rs for quantitative comparison with S2Rs of the same individual, a few much-stimulated specimens responded relatively often to the first flash of signal pairs. For example, female 62, featured in Figure 6, gave many S1Rs during the 140 minutes' exposure to 916 phrases (1932 stimulus flashes). Figure 7 shows the frequency distributions of the intervals between each signal and the first following flash. *Taken at face value* there were about 350 S1Rs, with latencies concentrated between 700 and 1800 ms (Fig. 7A), and 250 S2Rs distributed

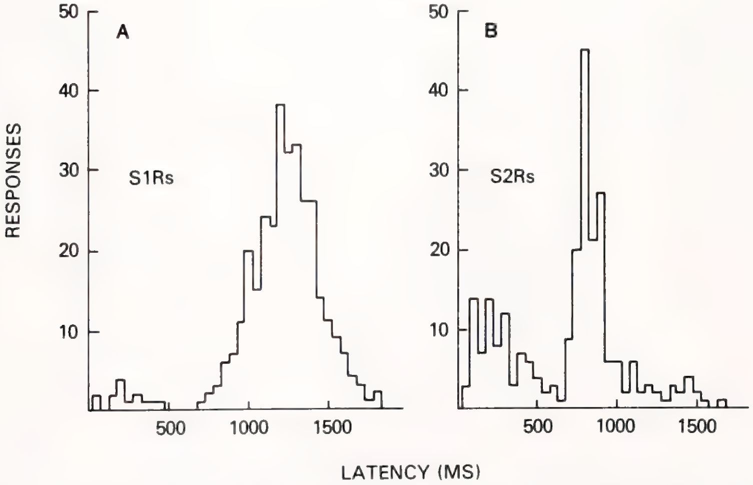

FIGURE 7. Frequency distributions of *prima facie* S1R (A) and S2R (B) latencies of female 62 during 916 cycles of continuous rhythmic driving.

between two populations, one peaking between 700 and 1000 ms, the other concentrated [illegible] below 300 ms (7B). The overall record included several quite uniform runs [illegible] or more consecutive S1Rs in the 1100 to 1200 ms latency range and a like [illegible] runs of 10 or more consecutive S2Rs in the 700 to 850 ms range, so there [illegible] little doubt that there do exist valid photic responses to both S1 and S2. It also seems safe to conclude from Figure 7 that any flash that follows a signal by more than 2000 ms is not a response but a spontaneous emission.

7. *Further S1R/S2R interrelations. Implication of spontaneity.*

In many experiments, under a variety of conditions, only S2Rs occurred. The latencies of these thousands of responses averaged 650–950 ms and were almost never shorter than 600. There is thus *a priori* reason to suspect that the 50 to 600 ms apparent S2R latencies in the above S1R-rich experiment (Fig. 7B) were not conventional responses to S2. A few flashes might have been spontaneous emissions that fell fortuitously within S1R or S2R latency ranges, but we believe most belonged to one or other of the following two effects, neither of which involved true S2Rs.

Because of the 400 ms minimal central nervous delay (Introduction) the short latency responses in Figure 7B could not have been evoked by S2s. Assuming that central delay was often longer than 400 ms, many could have been S1Rs. Thus (Fig. 8) any S1R excitation in which the centrally delayed fraction (CND) had been completed, and motor excitation (M) had started on its way to the lantern before the S2 signal arrived, would evoke a flash that could be misinterpreted as a short latency S2R.

The other phenomenon that almost certainly contributed to the spurious short-latency S2Rs of Figure 7B was the marked tendency for Female 62's flashes to occur in pairs about 1500 ms apart (Fig. 9). In about a third of the 90 such pairs recorded during more than two hours of unbroken driving, neither flash occurred less than 2 s after the preceding S2. By the criterion of Figure 7 such pairs were spontaneous. One such pair preceded each of phrases 4, 5, and 6 of Figure 6H and another is shown in Figure 9A. In another 30 instances either the first flash of the pair was spontaneous (*e.g.*, Fig. 6H, episodes 2, 6; 6I, episode 11; Figs. 9B–D) or the second was (Fig. 9I). In the remainder of the pairings, another 30 out of the 600 response total, it was not possible to distinguish between real S1Rs and flashes that happened to fall at a possible S1R latency (*e.g.*, 6A, episode 4; Figs. 9C–G). Similarly, S1RS2Rs (6C, episode 27; 6J, episode 17; 9E) were ambiguous in the sense that it is not excluded that the flash-pairing circuit might sometimes be activated by S1 rather than by the usual endogenous signal.

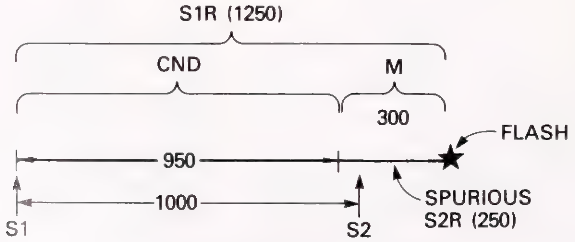

FIGURE 8. Proposed mechanism of misidentification of S1R as S2R. If excitation from S1 has completed its central nervous delay phase (CND) and triggered the motor outflow (M) to the lantern, a subsequent S2, before the flash, can be erroneously considered to be the effective stimulus.

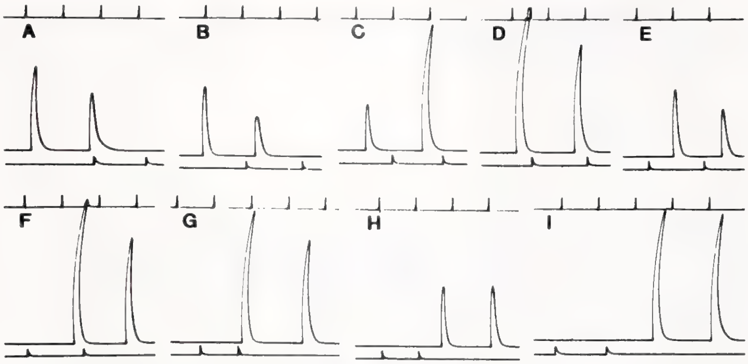

FIGURE 9. Flash pairs associated with paired signals of stimulus phrases. The types of association varied from one in which both firefly flashes were unequivocally spontaneous (SPSP; A), through instances in which the first flash was spontaneous (SPS1R; B, C, D) and the second not an S1R (B) or possibly so (C, D), to instances where both flashes might have been responsive (S1RS2R; E, F) or only the first (G, H), or probably neither (I).

Regardless of flashes of uncertain origin, the number of unequivocally spontaneous flash pairs, and the fact that they so often seemed independent of S1S2 scheduling and period, argue for flash pairing as a specific mode of female endogenous flash timing. Pair interflash durations for the entire record were heavily concentrated around a mean of about 1500 ms (22°) regardless of which of the 4 nominal classes of "response" was involved (Fig. 10). The validity of a spontaneous flash-pairing phenomenon was supported also by the flashing of one of the females in a slow driving experiment (Section 2, above). In this instance spontaneous flashing occurred throughout many of the 30 to 45 s rest periods between the isolated phrase presentations, much further removed from possible signal influence than with the 5 s and 10 s rhythms of the Figures 6–10 experiment, yet showed a pronounced 1500 ms (1.5 s) interflash peak (Fig. 11).

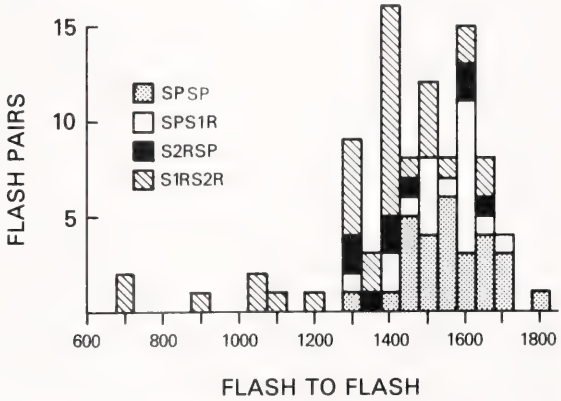

FIGURE 10. Frequencies of 90 interflash durations in four types of association of paired firefly flashes with paired signal phrases (*cf.* Fig. 9). The interflashes showed a strong independence from signal timing, suggesting spontaneity. Mean firefly interflashes: SPSP, 1530 ± 115 ms; SPS1R, 1543 ± 103; S2RSP, 1450 ± 135; S1RS2R, 1332 ± 243.

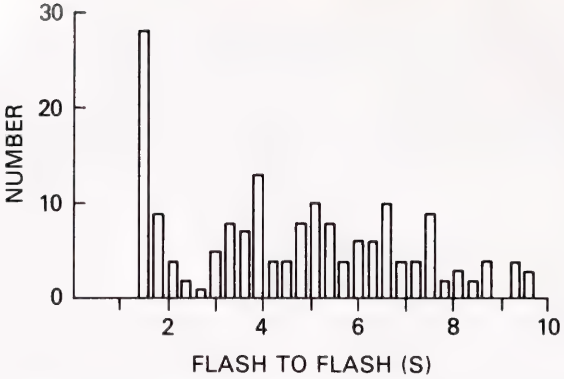

FIGURE 11. Frequencies of 124 spontaneous-flash-to-spontaneous-flash interval durations during about fifty 30 to 40 s intervals of non-stimulation, showing marked peak at about 1.5 s (1500 ms). Female 73.

In sum, though response overlap (Fig. 8) and flash pairing artifacts (Fig. 9) can account for the aberrantly short latencies of Figures 7A and 7B, and very probably some of the long latency tail of Figure 7B as well, photic stimulation of the female clearly can evoke a true response to the second stimulus of a phrase, after a delay of 700–950 ms at 22° (Fig. 7A) and sometimes also, or alternatively, a response to the first flash (or to a single stimulus flash) after a delay in the 800 to 1700 ms range, peaking at about 1250 ms (Fig. 7B). The S1R is thus not merely a premature S2R. How the two responses may be related mechanistically will be considered in the Discussion.

8. *Further endogenous flashing types*

In the field, neither unstimulated females nor those in dialog with either a male or with a flashlight were observed to flash spontaneously. Also, we recorded essentially no flashing during a 24-hour continuous box run with 4 unstimulated females. Enhanced spontaneity is probably a symptom of changing excitability induced by artificial driving, as is suggested also by the concurrently increased frequencies of flash skipping and of S1Rs. A major interest of spontaneity is that it supplements photic driving as an indicator of endogenous timing circuits. Flash pairing, for example, is clearly timed by an element with a relatively fixed duration averaging about 1500 ms at 21–22°. Evidence for other timed intervals or rhythms is more tenuous. The spontaneous flashes shown in Figure 6E suggest a rhythm period of about 5 s (mean of 6 interflashes = 4.8 ± 1 s), and the 4 spontaneous flashes interspersed between signal pairs 7 and 11 of Figure 6I divide that 40 s span into 8 intervals averaging 4.7 ± 0.4 s in duration.

Serially stimulated animals occasionally gave indication of endogenous luminescence at intervals in the 200–300 ms range. Usually this took the form of one full intensity flash preceded or followed by a much less intense shoulder, but occasionally the emissions were nearly equal in intensity (*e.g.*, Fig. 5H). In our opinion these emissions are not comparable to flash pairing. Rather, by reference to other fireflies, particularly during and after electrical driving (*e.g.*, Case and Buck, 1963; Buck *et al.* 1981A), they seem likely to be due to reverberation in a peripheral ganglion. Such short period emissions, and the fact that responses to rhythmic electrical driving may reach a frequency of several flashes per second (Case and Buck, in prep.), show that whatever it is that sets the minimal 2.5 s S2 to S1 limit to re-excitation by rhythmic photic signals (Fig. 5D) it is not physiological inability to flash faster.

DISCUSSION

Major flash timing patterns

Flash timing by *P. greeni* females results not only in the characteristic S2R response latency but in specific deviations related to photic history and stimulation regimen. When first exposed to paired flashes simulating the male's dialog code the female may not respond at all, then emit an irregular mixture of responses to the first signal of the pair (S1Rs), responses to the second (S2Rs) and flashes more than 2s later than the preceding stimulus (spontaneous flashes or SPs), then finally settle in to the repeated S2Rs of the natural dialog (Sect. 1; Fig. 2).

After warmup, many individuals continue 1:1 response for dozens or even hundreds of consecutive cycles if stimulus pairs recur at 15 s or longer intervals (Fig. 3). If pairs are presented more frequently, runs are typically shorter but still may include 1:1 rhythmic response (Fig. 5A, B). When the interval between successive pairs is 3.7 s or less, females no longer follow cycle by cycle, though they may respond at regular intervals (Fig. 5C, D). There may also be progressive falloff in flash intensity and increased frequency of response failures without change in stimulation format. Even during long stimulation series in which phrases are far enough apart to elicit 1:1 entrainment, response-skipping, S1Rs and SPs may develop, often alternating with runs of regular S1Rs or S2Rs (Fig. 6). A proper S1S2 interval is thus not enough in itself to assure indefinite rhythmic response.

S1R and S2R latencies differ in average duration (Fig. 7), and some apparent S1Rs and S2Rs are almost certainly spontaneous (Figs. 9, 10). There appear to be three specific categories of central nervous delay, associated with S1Rs (800+ ms), S2Rs (400+ ms) and endogenous flash pairing (*ca.* 1500 ms).

Types and causes of variability

There are several plausible reasons why identical stimuli did not always evoke the same response. Single cycle failures preceded and followed by dozens of consecutive responses (*e.g.,* Fig. 3, cycle 83, top trace) are presumably examples of the stochastic "noise" seen in most repetitive physiological events. In addition, latency, period and other response parameters of a given female of course exhibited statistical variation even during uniform rhythmic driving (Tables I, II).

Other types of response variation imply differences and changes in excitatory "state," a standard descriptive rubric for otherwise unexplained differences in responsivity to a given stimulation regimen. Thus the almost clocklike flashing of females stimulated in a slow rhythm (Fig. 3) illustrates a stable state of excitability lasting more than an hour. Alternating runs of S1Rs and S2Rs (*e.g.,* Fig. 6B–D) can be ascribed to an underlying cyclical variation in state. Females emitting sporadic spontaneous flashes may be presumed to be in a hyperexcited state. Fatigue is a likely cause of the progressive but reversible falloff in flash intensity during short-period rhythmic driving (Fig. 5D). The gradual decrease in response percentage that was common late in long rhythmic runs suggests a progressive falloff in general excitability, as in habituation. Functional recruitment during the warmup syndrome (Fig. 2) resembles arousal from the daylight torpor of *Photuris versicolor* (Case and Buck, 1963) which seemed to involve an actual physiological inability to flash and had to be overcome by mechanical agitation. It is presumably allied to the marked circadian rhythm of spontaneous flashing demonstrated in *Photinus pyralis* (Buck, 1937A).

One aspect of excitation that was difficult to quantify was persistence of response for one or more cycles after stimulus parameters were changed, or delay in resuming response when it had been interrupted temporarily. In Figure 6E, for example, the female flashed at 5 s intervals for 6 or 7 cycles after the stimulation interval had been lengthened to 10 s. Here one could invoke "memory" of the prior driving rhythm, persisting for several cycles without reinforcement. A possibly analogous behavior has been reported by Carlson *et al.* (1977) for *P. macdermotti* in that "Females can be tricked into answering a signal pair, the signal interval of which [S1S2] is usually non-stimulating, by preceding the out-of-range pair with signal pairs within her acceptable range." Also if *P. macdermotti* females had responded to repeated 2-flash patterns and then were given only the first flash of the pair, they responded at the time corresponding to a normal S2R (Lloyd, 1984; see also below).

Finally, the records amply confirm the existence of much individual variation in type and in timing of both responsive and endogenous luminescences. Different females run simultaneously under essentially identical conditions sometimes differed in response latency by as much as 15% (Table I, II) and in response percentage by even more, confirming Carlson and Copeland's (1978) denigration of stereotyped uniformity in firefly behavior.

An uncontrolled factor in response variability was the presumably non-uniform population of subject fireflies, a difficulty we tried to minimize by making a large number of tests on many females. All animals used were responsive at the time of collection, but differences might still be expected in view of our indiscriminate sampling. Some females remained responsive for up to 10 days in the laboratory, and presumably in nature. Hence, even freshly gathered individuals may have spanned considerable ranges in age, health, and reproductive history.

In view of the sometime lability of light emission and the departures from dialog format, it is necessary to emphasize that the singly-flashed S2R is not only the nearly exclusive response under field courting conditions but also overwhelmingly the characteristic response during laboratory series. In many thousand box tests on hundreds of females, regular 1:1 S2Rs were the usual response to either isolated signal pairs or pairs repeated at a moderate rate. Anomalous flashing and breakdowns in entrainment were interesting for their possible physiological and behavioral implications (see below) but did not cast any doubt on the validity and generality of the standard courting code.

Flash control model

The responses evoked by photic stimulation raise the question of what sort of central nervous control system might be responsible. To explain the S2R the control must account for the following: (1) the flash-evoking S2 signal is preceded by an identical signal (S1) that has no visible effect prior to S2 but is nevertheless essential to the S2R; (2) the S2 must occur within a restricted range of intervals after the S1 (Fig. 4); (3) the female's response flash occurs only within certain time limits after S2 (Tables I, II); (4) after the S2R, the system cannot be re-excited for at least 2.5 s (Fig. 5D, S2s to next S1s). In addition, the control must accommodate the variations in behavior that have been ascribed to differing levels of neural excitatory state and to individuality. A system that meets most of these requirements is diagrammed in Figure 12A. For simplicity the model assumes that both the initial visual input link and the final motor outflow are all-or-none processes of fixed latencies that trigger downstream

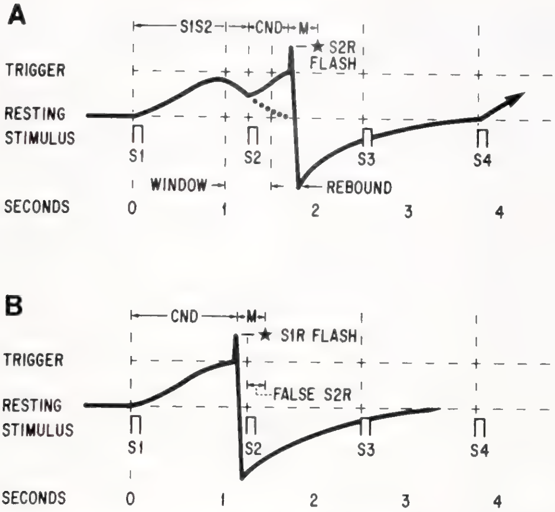

FIGURE 12. Model of female central nervous flash-control timer. A, S2R evocation. Excitation level rises after S1, then falls, enters the susceptibility window and rises again after S2, reaching the triggering level (at *ca.* 1750 ms) and exciting an S2R response. After the triggering spike, excitation level drops precipitously to a "hyperpolarized" level (REBOUND), from which it recovers so slowly that no excitation is elicited by S3. S4, arriving 2.5 s after S2, serves as a new primer for another response, after S5. S1S2 = 1250 ms; S2R = 800; CND = 500; M = 300; window = 500. B, S1R evocation. Excitation from S1 rises to triggering level at about 1100 ms, exciting a flash, then falls to a low level inexcitable by S2 or S3. The S1R flash at 1400 ms is also a spurious 150 ms S2R. S1S2 = 1250 ms; CND = 1100.

events but do not modulate the timing of light emission. The nominal 1250 ms S1S2 timing is a compromise between our mean preferred intersignal value at 23–25° (Fig. 4) and Buck and Buck's (1972) figures for 27°.

In the proposed model (Fig. 12A) some cellular excitation function (such as membrane potential or pulsatile activity within a group of central neurons) is represented as being at resting or basal level prior to S1. Upon S1 reception, excitation level begins to change but does not attain flash-triggering and falls back partway toward baseline (dotted line). After S1 the system is impervious to additional exogenous input for about 1s (Buck and Buck, 1972) then enters a time window, lasting about 500 ms, during which excitatory state is again susceptible to exogenous input. If S2 is presented during this window, excitation rises again, this time reaching the triggering level, thereby activating the motor signal to the lantern and evoking the S2R flash (star). Thus the S1 serves a trophic or priming function, raising excitation enough that an additional signal (S2) can achieve triggering. A female, after responding to S2, is refractory to photic input for at least 2.5 s (Fig. 5C, D). This could be accounted for if, after triggering, excitation falls below the resting level and occupies several seconds of central delay in recovering (Fig. 12A, "rebound").

Assuming that rate of increase in excitability can vary, the model provides plausible explanations for various departures from the standard S2R response. Flash skipping (*e.g.*, Fig. 2, episode 42; Fig. 3, episode 83; Fig. 6B, episodes 12, 22) would occur if excitation after the S2 did not rise to the triggering level. Similarly, if excitation after the S1 did not, as usual, peak and decline without reaching the triggering level (Fig. 12A) but continued to rise, an S1R could result (Fig. 12B). In that event, extra time would be occupied in the additional rise, and S1R latency would typically be longer than S2R latency, which starts from a higher excitation level. In support, the average S1R incorporated about 400 ms more central nervous delay than did the average S2R (Fig [illegible]

[illegible] model may also be compatible with certain non-dialog luminescences. A [illegible] spontaneous flash, for example, could be the analog of the S1R, with endogenous triggering in place of exogenous, or of the S2R, assuming that the endogenous timing control provides equivalents of both S1 and S2 signals. Spontaneous flash pairs, however, do not fit the model unless it is modified to provide (not unreasonably) that endogenous triggering does not induce the inhibitory undershoots proposed to follow response to external signals. Such a provision might also help explain the rarity of the two laboratory tandem events, S1RS2R and S2RS3R (Figs. 5G, 9, 10), already suspected of involving one or more spontaneous flashes.

The rate of rise of the transient should vary statistically and might also be subject to both endogenous and exogenous influences. Variations in response latency in the same female at different times and between different females run simultaneously (Tables I, II) should therefore be expected because the rates of charging and discharging of the timer would vary from cycle to cycle. The thresholds for basal and triggering excitation levels also would be expected to vary.

If the model is to accommodate the 300 ms latency to direct electrical eye stimulation (Introduction), it is necessary to envisage an immediate post-stimulus rise of excitation to the triggering level with consequent by-passing of central nervous delay.

In sum, an excitability transient operating between variable resting and flash-triggering limits (Fig. 12) provides a possible interpretation of the physiological control of the major female responses to photic input. Except as inherent in excitable membrane systems, the model does not supply specific physical bases for features such as the resting and triggering levels of excitability, the initiation and termination of excitation, or the post-S1 sensitivity window which is the key to the female's ability to count to two, *i.e.,* to recognize the male's characteristic flash pair code. In only one firefly has the flash-control area of the brain been even roughly localized, let alone analyzed (Bagnoli *et al.,* 1976, in *Luciola lusitanica*). Further elaboration of our model at this time would thus be gratuitous, particularly in view of the strong likelihood that flash control depends on a complex network of neurons rather than a single excitable cell. The model suggests an initial search for elements that are refractory for hundreds of milliseconds or more after excitation, and after flash triggering.

Behavioral comparisons, implications, and questions

Whatever the physiological basis of firefly laboratory flash timing phenomena, their behavioral significance in nature, and, *a fortiori,* their evolutionary implications, can only be established by exhaustive field observations. Such functional suggestions as we venture below are thus heuristic and tentative.

The sometimes marked individual differences in female flash-timing raises the question of how much variation is tolerated during the infrequently measurable natural dialog. Field data from several species show that the natural rhythm of the flying male's flashing varies in the same ranges observed in the present laboratory measurements on females (Buck, 1937B; Edmunds, 1963; Papi, 1969; Buck and Buck, 1972; etc.). It is also characteristic of firefly dialog that missed or mis-timed signals do not cause an immediate break in communication: males typically patrol the site of female answers for several cycles after response is interrupted, as it sometimes is by intervening foliage or by female failure to respond, and females often remain responsive indefinitely. It appears, therefore, that most timing variations measured in this study would not prevent the *P. greeni* female response to the male's flash pair nor male recognition of her response latency. If for no other reason, a dialog pattern used in situations in which the body temperatures of the respondents might be as much as 2° different at their respective distances above the ground must allow considerable leeway in signal timing.

Consistent differences in response latency (Tables I, II) in different females conceivably could supply a basis for individual recognition (Lloyd, 1984)—if males are capable of the necessary discrimination.

In typical *Photinus* firefly courtships the male is the active advertiser and the female a responsive, not signal-initiating, partner. It is therefore not clear how spontaneous flashing by the female of *P. greeni,* if it occurs in the field, could function in communication. Speculation is unjustified until it is found whether the male ever responds to single flashes not timed to his S2, or to flashes paired at his own intraphrase interval.

Lloyd (1969) and Carlson *et al.* (1976) described some *P. macdermotti* males as giving 2-2-2 second rhythmic single flashes when patrolling, and 2-4-2-4 second separated pairs when in dialog with a female or flashlight. This observation implies that the female normally responds both to unpaired flashes and to phrases and that the male is attracted to either answer. On the basis of still incomplete field observations and flight-cage experiments to be described elsewhere, the *P. greeni* male practically never emits unpaired flashes in flight but does sometimes approach S1Rs that follow several S2Rs from the same site. The S1R thus does not appear to play the systematic part in the *P. greeni* dialog that is reported for *P. macdermotti.* It might have utility in maintaining dialog in underbrush, where the male's S1 flash must sometimes be occluded (*cf.,* Carlson *et al.,* 1976; Lloyd 1984).

The fact that both males and females of *P. greeni* can emit pairs of spontaneous flashes deserves special comment. Usually, apparently, paired flashes are emitted only by the male. However, the female's response to the male's phrase is proof that her flash control system includes a timing element tuned to the S1S2. In view of the temperature coefficient of 2+ for flashing parameters (Materials and Methods), the female's mean 1500 ms intra-pair emission interval at 21–22° (Figs. 10, 11) and the male's field advertising rhythm at 27° (means for two individuals, 1260 and 1330 ms, Buck and Buck, 1972) should be close enough at a common temperature to suggest that they are generated by homologous timing circuits. Given that male and female are almost identical genetically it seems reasonable that in certain excitatory states the female's normally *sensory* timer could *initiate* flashes, as in the male. Somewhat analogous situations have been reported by Hoy *et al.* (1977) and by Doherty and Gerhard (1984) in the calling signals of crickets and frogs, respectively.

One additional gain from exploring the limits of flash-control is speculative insight into the evolution of firefly neural timing systems. Fascinating though such questions are, they are subject to the general caveat that any change in motor output-timing (male flashing period; female latency) would require concurrent change in the corresponding sensory input-timing of the other sex (Buck, 1978). Hence, simply being able to imagine a selective pressure for a code transition does not necessarily translate into a physiologically reasonable mechanism, or *vice versa.*

Lloyd (1984) reported that 13 late fall *P. macdermotti* females, if primed with signal pairs simulating the male's 2 s phrase, almost always responded to a single stimulus ("P1") after a delay of 3 s rather than at their normal 1 s latency to P2. From this evidence Lloyd proposed that the present courting code of *Photinus ignitus,* a close relative of *P. macdermotti* and *P. greeni* with a 3 s female response latency to the male's single signal, evolved by dropping out the second flash of the male's phrase of a *macdermotti*-like ancestral species. The *P. ignitus* P1R would thus correspond not to the *greeni* S1R but to S1S2 + S2R. Assuming that *macdermotti* has the same flash-control system as *greeni,* Figure 12 suggests no obvious way to couple the female's S1R central delay to the equivalent of the S2R latency unless flashing at the end of the phrase-timing circuit can be triggered endogenously as well as by S2 (P2). In *P. greeni* we have not observed such a response, which would need to have a mean latency of at least 2 s, although we have not tested single signals as extensively as Lloyd.

Space does not permit discussing the three selection rationales proposed by Lloyd for the evolution of the *P. ignitus* code, but to us they seem no more persuasive than the view that evolution has proceeded in the opposite direction—that is, from codes in which the male emits a single signal to those in which he flashes in timed pairs. The selective advantage of a flash pair over a single flash would be the great increase in the female's ability to identify the male of the species. As shown by the ease of signal simulation with a wide variety of electric lights, neither flash intensity, flash duration, nor male advertising period offers a good basis for discriminating a conspecific single flash from singles of other species, or even other females. In that sense the *P. greeni* S1R might be the atavistic echo of a simpler past code, still latent in the nervous system.

In sum, exploration of *P. greeni* female responses under as natural conditions as compatible with controlled photic stimulation both confirmed the communicative pre-eminence of her response latency to the second flash of the male's courting phrase and yielded robust examples of systematic departure from standard dialog.

Regardless of whether our draft model proves to be correct in principle and whether particular timed intervals have a regular role in field behavior, the experiments revealed something of the range and potentialities of flash-control behavioral physiology. Spontaneous flashing, response skipping, S1Rs and other infrequent behaviors are *bonafide* capabilities of the intact nervous system. Some may serve only rarely in a communicative role, others may reflect response modalities used before the specialized dialog code had evolved to its present state, or indicate that similar underlying timing circuitry exists in the nervous systems of both male and female, as in the normal male and induced female emission at *ca.* 1500 ms intervals and possibly at the 5 s motor rhythm. Still others may be the physiological consequences of unusual input. Such responses, and the ability of the unrestrained, isolated female to produce spontaneous flashes, warn that not all nuances of light emission need be part of dialog. In fact there is good reason to expect that neural noise and fluctuations in excitability can and do normally provide a variety of occasional flashing behaviors that have no positive communicative significance.

ACKNOWLEDGMENTS

We are grateful to Albert Carlson, Jonathan Copeland, John Hildebrand, Ronald Hoy, Ladd Prosser and anonymous reviewers for helpful criticism, and to Betty Morris for multiple retypings.

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ORMAL GAMETOGENESIS AND SEX RATIO IN TRIPLOID SOFT-SHELL CLAMS (*MYA ARENARIA*)*

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ABSTRACT

Triploid soft-shell clams (*Mya arenaria*) were produced by inhibiting polar body extrusion with cytochalasin B immediately after fertilization. Diploid and triploid clams grown in suspended tray culture were examined histologically during the reproductive season of their second year. Most diploids had matured by the end of May and displayed normal gametogenic development in every individual. Triploids did not mature and most had undeveloped gonads. The few triploid females which developed some oocytes by the end of May had abnormal maturation. Triploids were 77% female as judged by the presence of oocytes; another 16% were female-like and may have been intersexes; the sex of 6% could not be identified because there was no sexual differentiation. Sex determination in this species, based on the sex ratio in triploids, best fits the model of an X:autosome balance mechanism as exemplified in some insect species.

INTRODUCTION

Mya arenaria is a deep-burrowing infaunal bivalve found ubiquitously on coastlines of the Northern Hemisphere. It is the basis of a large commercial fishery along the East Coast of North America. Recent efforts to replant clam flats led to investigations into the feasibility of producing and seeding triploid clams.

The utility of induced triploidy to aquaculture or management is founded on the supposition that triploids are sterile (Thorgaard and Allen, in press). In several species of teleosts, triploid males characteristically develop small testes and females have rudimentary ovaries (Thorgaard and Gall, 1979; Lincoln, 1981a, b; Wolters *et al.*, 1982; Yamazaki, 1983; Benfy and Sutterlin, 1984). The production of all-female triploids has thus been embraced as a guarantee of sterility (Lincoln and Scott, 1983). Sexual maturation is inhibited in both sexes by the arrest of gametogenesis in the gonial stages. The sex ratio of triploid teleosts does not depart from the 1:1 ratio expected of a homogametic female sex determining mechanism (Swarup, 1959; Gervai *et al.*, 1980; Wolters *et al.*, 1982; Yamazaki, 1983) except in the case of triploid plaice: Lincoln (1981b) reported 20 males and 1 female.

Induced triploidy in shellfish has only recently been described (Stanley *et al.*, 1981; Allen *et al.*, 1982; Chaiton and Allen, 1985) so comparisons of diploid and triploid gametogenesis are lacking. Tabarini (1984) produced triploid scallops, *Argopecten irradians*, and the majority (61% and 91% from two sampling locations) failed to ripen; 39% and 9% were reported as ripe or partially ripe. However the nature of gametogenesis

Received 15 April 1985; accepted 13 December 1985.

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in these functional hermaphrodites was not explored histologically. The purpose of our study was to examine the course of gametogenesis during the reproductive season in diploid and triploid soft-shell clams.

MATERIALS AND METHODS

Triploidy was induced in soft-shell clam eggs by inhibiting the extrusion of a polar body with cytochalasin B (Allen *et al.*, 1982). Fertilized eggs were treated with cytochalasin either from 0 to 15 or 15 to 30 minutes after fertilization. Experimental groups and a control group—treated only with DMSO, the carrier for cytochalasin—were reared at the I.C. Darling Center, Walpole, Maine, for two years. Triploids resulting from the two treatments were sampled at four intervals during the reproductive season (3/22/82, 4/12/82, 5/4/82, and 5/25/82) and were prepared for histological examination.

Triploidy was confirmed for all experimental clams except those samples on 5/4/82. Siphons were removed and frozen for determination of ploidy by electrophoresis (Allen *et al.*, 1982) or flow cytometry (Allen, 1983).

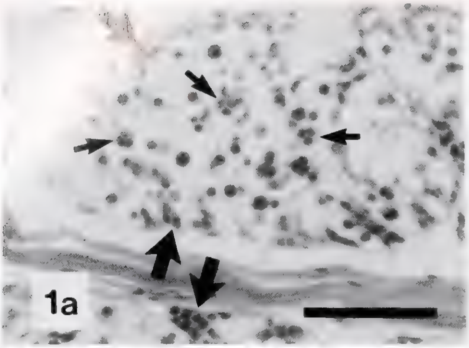
For histological examination of gonads the body of the clam was fixed for 12 hours in Helly's Fixative (Humason, 1979), washed 12–24 hours in 3% potassium dichromate, followed by a 12 hour wash in tap water before standard dehydration and imbedding series. Slides were prepared from tissues sectioned at 6 μm and stained in Heidenhain-Mallory-Azan, as described in Humason (1979). A total of 59 controls and 64 confirmed triploids were analyzed.

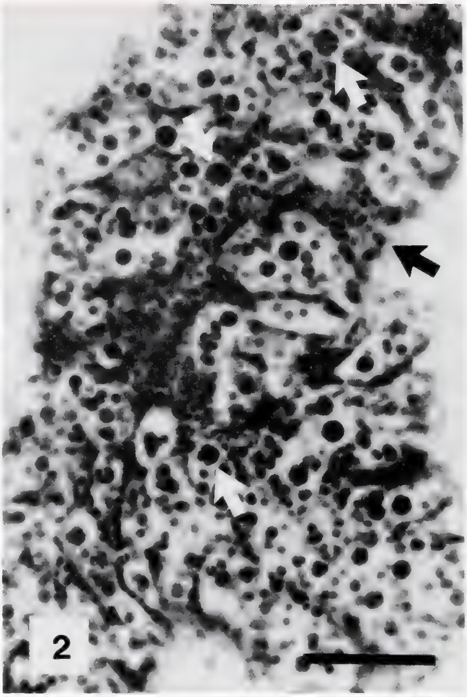
Gonadal maturation was assessed by assigning a state of maturity based on the degree of gametogenesis. This scale is similar to gonadal stages used by Shaw (1965), Ropes and Stickney (1965), and Porter (1974) who described diploid gametogenesis, with the following exception: we assigned an additional stage between that of Inactive and Early Active to add further resolution to the scale during early gametogenesis. Gonad formation in the soft-shell clam commences when the animal is as small as 6 mm (Coe and Turner, 1938). Early gonad formation begins with the proliferation of gonadal primordia to form "solid, cylindrical, profusely branching alveoli filled with large, vacuolated follicle cells and widely scattered gonia on the periphery" (Coe and Turner, 1938). Our analysis did not include sampling that would follow this development but there appeared to be no differences in the gross anatomical formation of the alveoli between diploids and triploids; the index below begins at the stage of sexual differentiation of the primary gonia at the periphery of the alveoli.

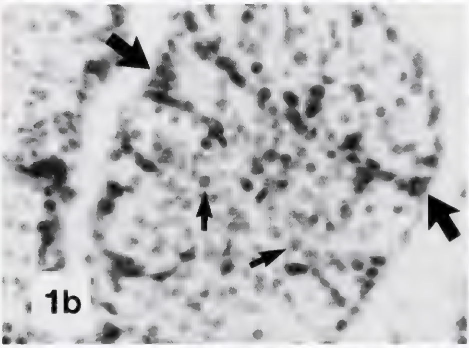
Males

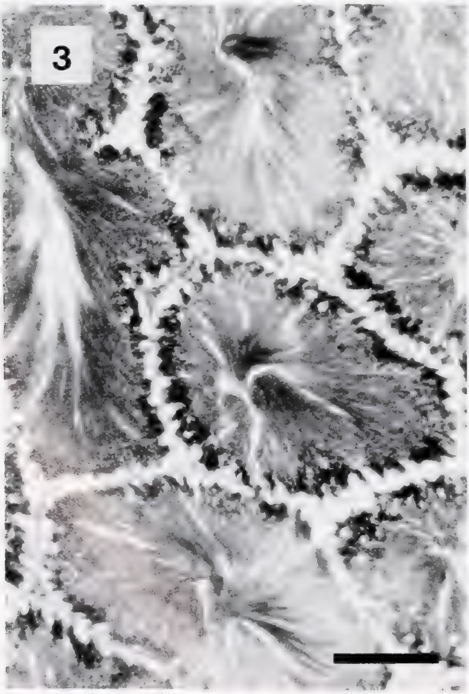
Inactive (Fig. 1a). Undifferentiated gonia are distributed around the periphery of alveoli and widely separated by vacuolated follicle cells which fill the alveoli. Follicle cells may have two types of inclusions. Lipoid-type nutritive inclusions, derived from the thin layer of cytoplasm which surrounds the central vacuole of the follicle cells (Coe and Turner, 1938), begin to accumulate at the time of sexual differentiation, and thus are not abundant in the inactive stage. Abnormal spermatogenesis results in a second type of inclusion characterized by multinucleated cysts. These may become pycnotic and nutritive when cytolyzed in later stages of gametogenesis. Aberrant spermatogenesis begins before the onset of normal gametogenesis (Coe and Turner, 1938) and therefore is not considered part of the active cycle.

Very early active (Fig. 1b). Follicle inclusions are most numerous when the differentiated primary spermatogonia begin to divide. The secondary spermatogonia which form are no longer widely separated by follicle cells.









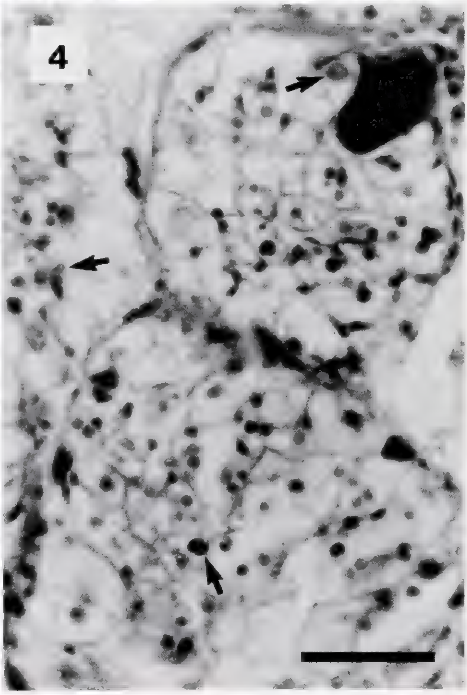


FIGURE 1a. Cross section of an alveolus in a diploid male clam in Inactive stage. Small arrows indicate multi-nucleated products of abnormal gametogenesis. Large arrows show spermatogonia. Bar = 50 μm. 250×.

Early active (Fig. 2). Primary and secondary spermatogonia proliferate in columns that invade the space occupied by the follicle cells and extend toward the developing central lumen of the alveoli. This intrusion of dividing gonia among the follicle cells characterizes mitotic divisions in the male clam. The number of successive spermatogonial mitoses may be six to nine (producing 64–512 spermatocytes), based on the range observed in other molluscan species (Roosen-Runge, 1977). Nutritive inclusions are still numerous as are products of abnormal spermatogenesis. Some spermatocytes accumulate in the center of the alveoli.

Middle active. A continuous gradient of cell types, ranging from spermatogonia at the basal membrane of the alveoli to spermatids (spermatozoa present in some alveoli) in the enlargened lumen, displaces nearly all the follicle cells. Spermatocytes and spermatids predominate. Nutritive inclusions, utilized as active gametogenesis continues, are less numerous.

Late active. Several rows of spermatogonia and/or spermatocytes remain along the basal membrane but the remainder of the alveoli are packed with radially aligned spermatids and spermatozoa. Follicle cells and inclusions are absent. The pink-stained flagella of spermatozoa extend into the central lumen of alveoli.

Ripe (Fig. 3). Alevoli are filled with radially aligned spermatozoa.

Female

Inactive (Fig. 4). As in the male, undifferentiated gonia are widely separated by follicle cells. Inclusions typical of the female include small male-type lipoid globules and larger elements, possibly albuminous, derived in part from the cytolysis of degenerating oocytes and in part from the activities of the cytoplasm of the follicle cells (Coe and Turner, 1938). Clams were identified as females by the presence of at least an occasional oocyte. We identified some indeterminate clams as probable females based on the appearance of differentiating gonia. Completely undeveloped clams could not be sexed—the primordial gonia cells and follicle inclusions in the alveoli had not proceeded to the stage of sexual differentiation.

Very early active. At least one oocyte is present in all follicles but little growth has occurred. Intact follicle cells fill alveoli.

Early active (Fig. 5). Many oocytes begin to grow (= auxocytes) and elongate toward the center of the alveoli, although lumen have not formed. All oocytes are still attached to the basal walls of alveoli. The follicle cells contain nutritive inclusions.

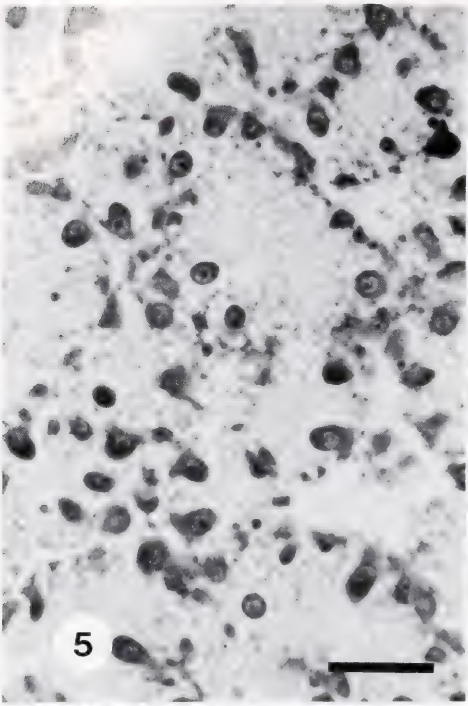
Middle active. Follicle cells break down, forming lumen with occasional free ova. Most of the enlarged oocytes are still attached by a slender stalk and most or all nutritive inclusions have disappeared.

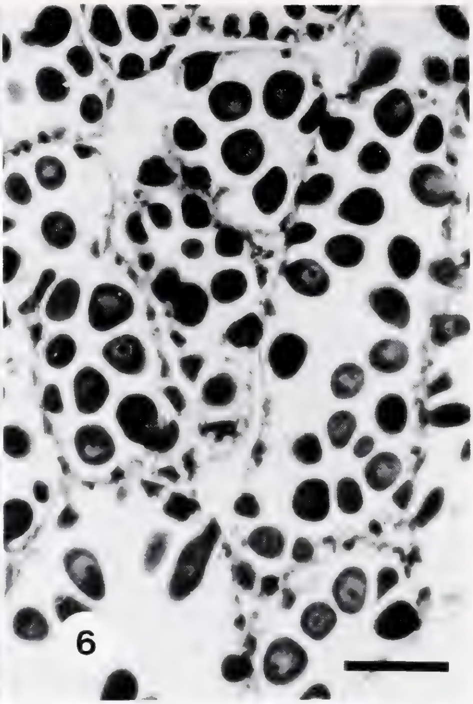
FIGURE 1b. Alveolus of Very Early Active diploid male clam shows spermatogonia which have undergone several mitotic divisions (large arrows). Divisions proceeded perpendicularly to basal membrane of alveolus and invaded the space occupied by the follicle cells. High numbers of inclusions (small arrows) can be seen in follicle cells. 250×.

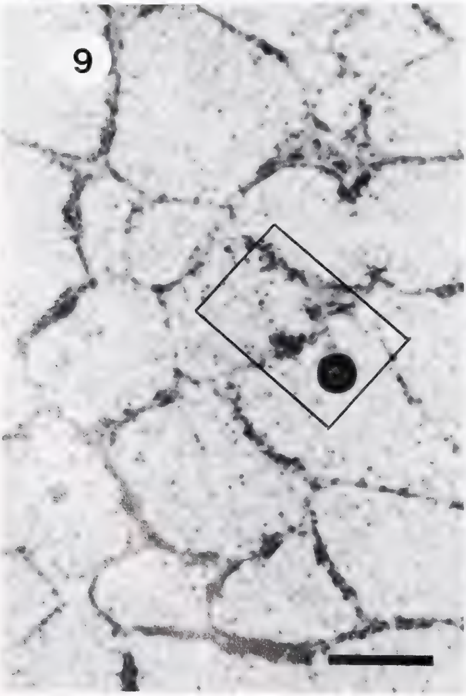
FIGURE 2. Early Active diploid male. Mitotic divisions are now extensive. Secondary spermatogonia extend from foci at the basal membrane of the alveolus (black arrow) into the center where they will further differentiate in later stages. White arrow indicates products of abnormal spermatogenesis that still persist. Bar = 50 μm. 250×.

FIGURE 3. Ripe diploid male. Alveoli are filled with radially aligned spermatozoa. Bar = 100 μm. 100×.

FIGURE 4. Female diploid in Inactive stage. Few oogonia are present although one growing auxocyte enables classification as female. Inclusions (arrows) are not as numerous as in the male. Bar = 50 μm. 250×.







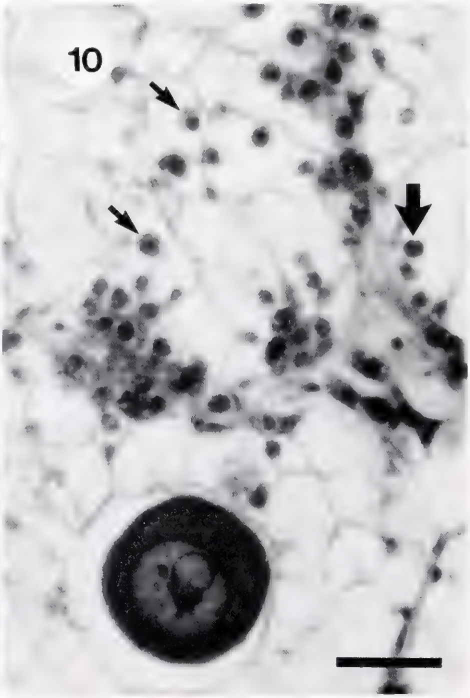


FIGURE 5. Early Active diploid female. Oogonial divisions are not normally very extensive before many developing oocytes can be seen. Bar = 100 μm. 100×.

FIGURE 6. Ripe diploid female with many free ova in alveoli. Bar = 100 μm. 100×.

Late active. Many ova have reached maximum size and are free in central lumen of alveoli. Those oocytes still attached by a slender stalk are more spherical with clearly visible nucleoli and amphinucleoli.

Ripe (Fig. 6). Most of the ova are no longer attached to the basal membrane of the alveoli. Follicle cells are absent.

RESULTS

Proportion of triploids

When this same population of clams was sampled at 8 months (Allen *et al.*, 1982) 89.5% were triploid (n = 86) in the group treated from 0–15 minutes after fertilization and 78.5% were triploid (n = 79) in the group treated from 15–30 minutes after fertilization. In the present study 83.7% (n = 43) of those in the first treatment and 66.7% (n = 45) of those in the second treatment were triploid. A Chi square analysis comparing the proportion of triploids at 8 months with that at 2 years shows no significant differences ($\chi^2 = 1.04$, $P > .50$ and $\chi^2 = 0.81$, $P > .50$, respectively), suggesting that there is no differential mortality associated with triploidy up to first spawning.

Gametogenesis

Diploid males and females did not mature until the last sampling period when all specimens were at or past the Middle Active period (Fig. 7). In March and April only one of the 14 females and 7 of 13 males were Inactive. By May 25 the diploid population as a whole was characterized as Late Active. The triploids were largely immature. During March and April 41 of 42 triploids were classified as Inactive (Fig. 8). On May 25, 15 of 22 were still inactive. Only two triploids reached Middle Active.

None of the triploid clams were unequivocally identified as males in any of the sampling periods. Four undeveloped triploids had no sexual differentiation, as judged by the types of inclusions or by the nature of the divisions of the primary gonia. These obviously were classified as Inactive. Of the other 60 triploid clams 49 were apparently females, with definite oocytes (Figs. 9, 10). Oogonia in triploids often had a peculiar proliferation with no apparent differentiation into oocytes, resulting in pockets or clusters of oogonia at the edge of the alveoli (Fig. 10). These clusters were localized and not characteristic of the columnar intrusion into the follicle cells that is typical of male gonads.

Ten triploids did not develop oocytes but the pattern of division of the gonia, and the amount and kind of inclusions were typical of triploid females (Fig. 11). In some cases gonia proliferation would nearly fill the alveoli. Many of the cells in these clusters may have been premeiotic oocytes, *i.e.*, with large, early prophase nuclei. This feature alone however is not sufficient to discriminate between oocytes and spermatocytes. Other than that one characteristic there was no way to tell the sex of these indeterminate individuals. These 10 triploids were probably female and may have been "female" intersexes. One triploid had an atypical cytology: some of the gonia at the basal membrane of the alveoli began to divide in a manner typical of male divisions, *i.e.*, divisions

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FIGURE 9. Female triploids could be identified because of presence of occasional auxocytes which were generally nearly ripe. Edges of alveoli were heavily outlined by oogonia which presumably have divided mitotically. There were relatively few inclusions in the follicle cells. Inset of Figure 10. Bar = 100 μm. 100×.

FIGURE 10. Inset from Figure 9 at 400× shows nests of oogonia from which no oocytes are differentiating. Large arrow—possible degenerating oocyte; small arrows—non-germ cell inclusions. Bar = 25 μm.

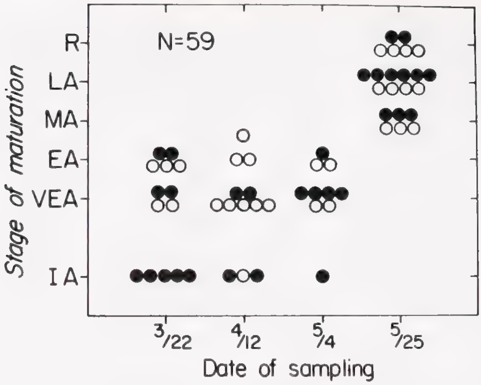

FIGURE 7. Stage of maturation (described in Materials and Methods) was plotted against sample date for males (closed circles) and females (open circles) in *diploid* clams.

progressed in columns perpendicular to the basal membrane and invaded the space occupied by follicular cells. Most of the inclusions, however, were typical of those seen in females. Furthermore non-germ cell inclusions were typical of Inactive female gonads of diploids. This individual was probably an intersexual "male."

A few females in the triploid group appear to have undergone normal or near normal early maturation (*i.e.*, five Very Early Active on 5/25). Another female (Very Early Active on 3/22) had maturing or mature eggs in the alveoli but few young oocytes. The maturation of most gonia appeared arrested. Note in Figures 12 and 13 that maturation categorized as Very Early Active and Early Active, respectively, had mature ova in the central lumen of the alveoli but few young oocytes were present. We believe that this condition was abnormal and indicative of reduced maturation. Also uncertain is whether the two Middle Active triploids observed on 5/25 (Fig. 14) were derived from further maturation of animals similar to those shown in Figures 12 and 13 or whether they represent normal maturity.

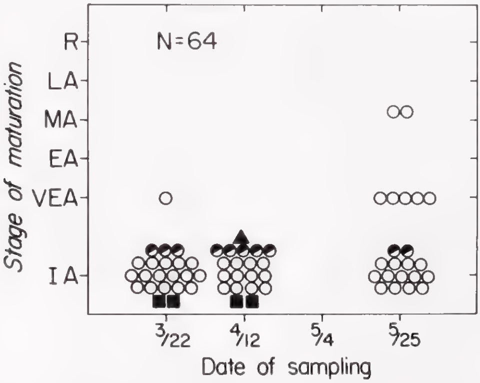

FIGURE 8. Stage of maturation was plotted against sample date for the various classes of *triploids* (described in Materials and Methods). Open circles—females; half-circles—indeterminate; square—undeveloped; and triangle—atypical cytology.

Inclusions

Follicle inclusions in the soft-shell clam serve as nutritive reserve for periods of rapid gametogenesis. Therefore the character and abundance of these substances are important to maturation. We created a scale for the abundance of inclusions based on the greatest abundance equalling 10 (found only in some males) and no inclusions—0 (as is the case in ripe or nearly ripe diploids). The values of these ratings in each sampling period were then tested non-parametrically using a two tailed Mann-Whitney test. The null hypothesis was that there was no difference in the abundance of intrafollicular inclusions between female diploids and triploids. There were significant differences between the abundance of inclusions in diploids and triploids (Fig. 15) on 3/22 ($U = 97.5$, $P < .05$), 4/12 ($U = 134.5$, $P < .01$, and 5/25 ($U = 146.5$, $P < .01$). In diploids, nutritive inclusions were accumulated at sexual differentiation and inclusions of all kinds were cytolyzed and used as gametogenesis proceeded. In triploids nutritive inclusions were relatively scarce at the beginning of the season, but because they were not utilized, they continued to accumulate. In addition products resembling those produced by abnormal gametogenesis in males also accumulated as the season progressed. By the last sampling period, triploids had significantly greater quantities of inclusions than diploids, or triploids in any other sampling period ($U = 336.5$, $P < .001$).

DISCUSSION

Mechanisms of triploid sterility

We have demonstrated that triploid soft-shell clams, unlike their diploid cohorts, do not mature sexually late into the spawning season. Reproductive sterility is a common feature of artificially induced triploids in vertebrates. In addtion to teleosts (see Thorgaard, 1983 for review) triploid sterility has been described in Anurans (Humphrey *et al.*, 1950; Kawamura and Tokunga, 1952; Sato, 1952; and Ueda, 1980) and Urodeles, although the latter group sometimes produces functional gametes (Griffiths, 1941; Kawamura, 1940; Fankhauser and Humphrey, 1950). There are relatively few accounts of triploid sterility in invertebrates. In this report the majority of triploid *M. arenaria* had inactive gonads based on the absence of maturing gametes. Oocyte development, when it occurred, was abnormal. Only two triploids reached the stage of Middle Active.

Spermatogenesis is completely abortive in naturally occurring races of triploid trematode flatworms (Terasaki, 1977; Sakaguchi, 1980; Sakaguchi and Tada, 1980; Ramanjaneyulu and Madhavi, 1984) and cestode flatworms (Sasada, 1978; Grey and Mackiewicz, 1980). In the majority of these species meiosis ceases because homologues fail to synapse. However, in one trematode species (*Glaridacris catastomi*), multivalents form in the spermatocytes and subsequent meiotic divisions are disrupted (Grey and Mackiewicz, 1980). Meiosis in female triploid flatworms is also asynaptic and consequently embryonation begins following a mitotic maturation division. In triploid *Drosophila* females, two of the three bivalents pair at zygotene leaving the third unpaired (White, 1977). With only four pairs of chromosomes, meiotic segregation produces a relatively high proportion of functional, viable gametes. Soft-shell clams have $2N = 34$ chromosomes ($3N = 51$) (Allen *et al.*, 1982), therefore either mechanism, asynapsis or multivalent formation/aberrant segregation, would result in abnormal gametogenesis. Because we observed a few oocytes that appeared to be normal auxocytes, perhaps some gametes "develop" via the latter mechanism.

The presence of clusters of seemingly undifferentiated gonia suggests that gametogenesis in the clam is arrested, rather than uninitiated. In some cases these clusters

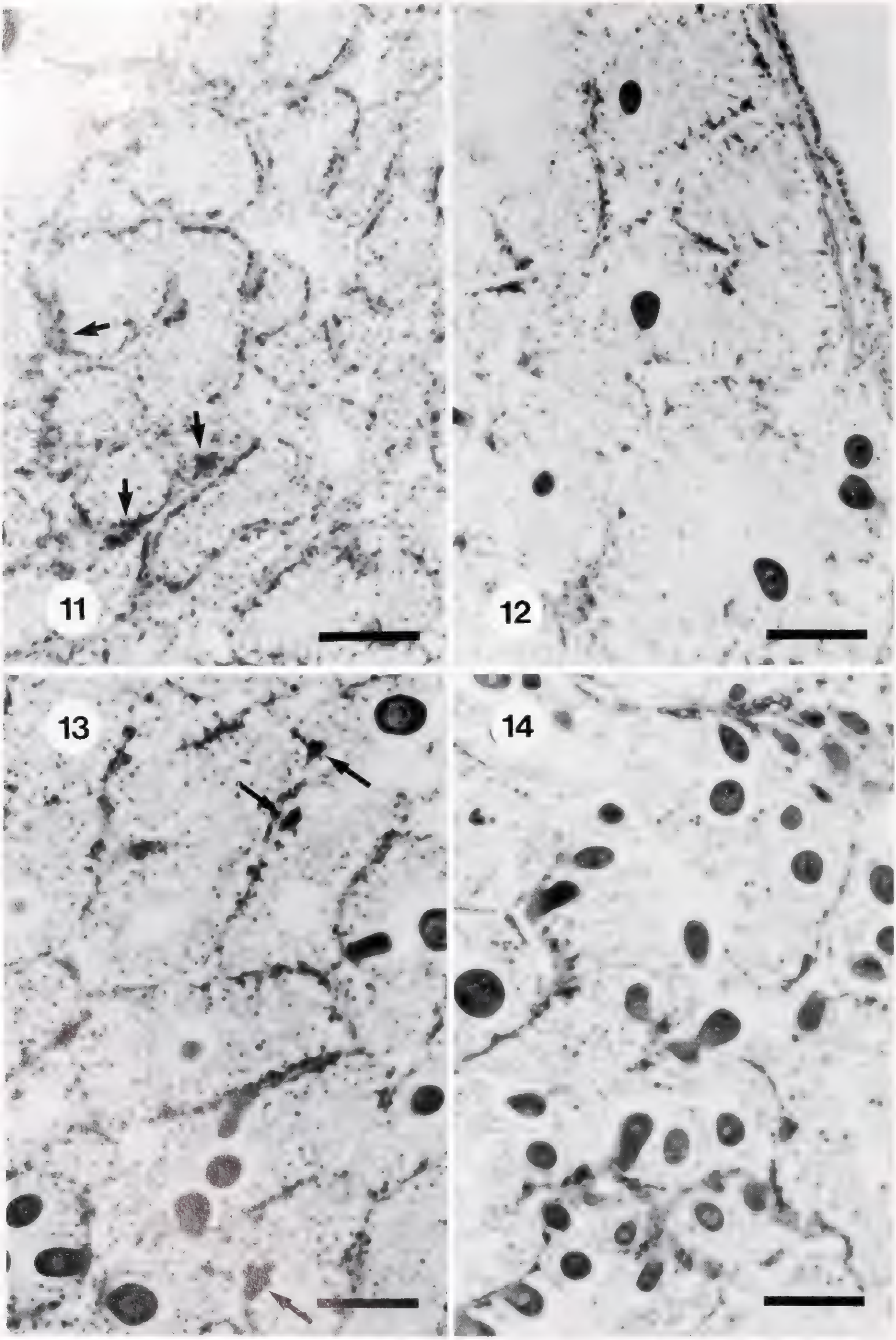

FIGURE 11. Indeterminate triploid "female" was characterized as such because of (1) the similarity in general appearance of gonads, *i.e.*, alveoli heavily outlined by gonia (nests indicated by arrows) and (2) the lack of similarity of these mitotic nests to typical male mitoses. Inclusions tended to be of non-germ cells. Bar = 100 μm. 100×.

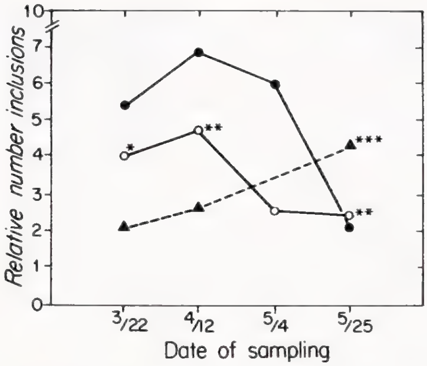

FIGURE 15. Relative number of inclusions were rated 1–10 in each specimen sampled. The means of diploid males (closed circles), diploid females (open circles), and all triploids (triangles) were plotted against sample date. Values of diploid females were tested by a Mann-Whitney test against the values of triploids, and triploids in 5/25 were tested against triploids in 4/12. *—$P < .05$; **—$P < .01$; ***—$P < .001$.

were extensive, with gonia cells nearly filling the alveolus. Lincoln (1981b) reported "nests of cells" in triploid plaice × flounder hybrids and attributed them to the persistence of secondary oogonia that had been arrested in meiosis. Gonia also proliferate in triploid *Rana pipiens* (Humphrey *et al.*, 1950) and *R. japonica* (Kawamura and Tokunga, 1952) but is followed by degeneration of premeiotic oocytes, and subsequently sex reversal (Humphrey *et al.*, 1950). Both oogonia (Raven, 1961) and spermatogonia (Roosen-Runge, 1977) undergo mitotic divisions prior to stages as oo- or spermatocytes. In diploid clams, spermatogonial proliferation was characterized by the invasion of secondary spermatogonia into the space occupied by the follicle cells; oogonial proliferation was not as extensive and was followed immediately by differentiation of premeiotic oocytes into auxocytes. Triploids identified as females (by the presence of auxocytes) shared characteristics of gross gonadal structure with triploids categorized as indeterminate (for lack of oocytes). It was this feature and not the premeiotic appearance of the gonocytes which led us to characterize indeterminates as "female" intersexes. Similarly we suggest that the clams identified as undifferentiated in early sampling periods would have "developed" into females, based on the *post facto* examination of the heavily skewed sex ratio.

Triploidy and sex ratio

We have demonstrated that at least 77% of the triploid clams are female, but 93% have histological features of females. Several correlations between the sex of triploids and sex determination can be drawn from the literature. Triploid teleosts, with one exception, have a 1:1 sex ratio confirming (Gervai *et al.*, 1980; Wolters *et al.*, 1982;

FIGURE 12. Female triploid classified as Very Early Active (VEA) although not typical of VEA in diploids. Only nearly ripe oocytes were present and (in this photo) no developing oocytes can be seen. If oocytes were not present this specimen would be classified as undeveloped. Bar = 100 μm. 100×.

FIGURE 13. Female triploid classified as Early Active because of presence of large oocytes. But as in the case of Figure 12, few young oocytes (arrows) are present. Alveoli have premature lumen. Bar = 100 μm. 100×.

FIGURE 14. Middle Active female triploid represents the most advanced stage of sexual maturity seen in the triploids sampled. Like the specimens in Figures 12 and 13 triploids displayed a tentative type of "maturity" if they began to develop at all. Figure 14 may be representative of the way some diploids mature although we saw none in our diploid samples. Bar = 100 μm. 100×.

Onozato, 1985) or suggesting (Swarup, 1959) a heterogametic male (XY) system. In triploids, XXY is male and XXX is female. In the one exception, Lincoln (1981b) could not account for an overabundance of triploid male plaice, a fish with a WZ (female heterogamety) sex-determining mechanism. The sex ratio in triploid *Rana* spp. (Anura) is variable. Triploid *R. pipiens* and *R. japonica* reverse sex from female to male prior to metamorphosis (Humphrey *et al.*, 1950; Kawamura and Tokunaga, 1952), while triploid *R. nigromaculata* show a preponderance (38:5) of males (Kawamura, 1941). However, females are most frequent in triploid *R. limnocharis* (Sato, 1952), and triploid fire bellied toads, *Bombina orientalis,* have a 1:1 sex ratio (Ueda, 1980). Males in these anurans have been shown to be the heterogametic sex (Ueda, 1980 and references therein); variability in the expression of sex ratio in triploids is apparently due to differences in the potency for sexual development mediated by the medulla of the gonad (Kawamura and Tokunga, 1952).

An alternative explanation was offered for the preponderance of females in the toad, *Bufo vulgaris formosa.* Muto (1952) suggested that the toad, like the urodeles *Triturus,* possess a WZ sex determining system. Furthermore, the second maturation division "may be reductional for W and Z," *i.e.,* following a crossover event (Muto, 1952). Suppressing this second division would yield WZ eggs, which, when fertilized by Z-bearing sperm, would produce a preponderance of females. Griffiths (1941) and Kawamura and Sanada (1949) observed mostly females in triploid *Triturus viridescens* and *T. pyrrhogaster,* respectively. However, Fischberg (1945) reported a nearly 1:1 female:male ratio in triploid *T. alpestrus.*

Sex determination in *Drosophila,* and probably in one or two branches in the phylogeny of winged insects (White, 1977), depends on the balance between the female-determining X chromosome and male-determining autosomes (A). Triploid 3X:3A individuals are all female; XXY:3A are intersexual—phenotypically intermediate between maleness and femaleness. Triploid "male" intersexes usually die but some differentiate testes which undergo abnormal spermatogenesis; XXY:3A ovaries produce mature oocytes in some cases (review by Baker and Belote, 1983). Triploid silkworms, *Bombyx mori,* develop both sexes with no intersexuality: the presence of Y (=W) in the heterogametic female is the dominant sex determining vector (Tanaka, 1953).

The genetics of sex development in the nematode, *Caenorhabditis elegans,* is particularly well studied. Normally a self-fertilizing hermaphrodite with an 2X:2A chromosome constitution, non-disjunction occasionally gives rise to X0:2A males. Triploid 3X:3A are hermaphrodites while 2X:3A individuals are male, a similar system to that of *Drosophila* (Bull, 1983). In both *C. elegans* and *Drosophila,* specific genes have been identified with major regulatory control over sexual differentiation (Baker and Belote, 1983; Doniach and Hodgkin, 1984). The products of this regulatory cascade are mediated by the "assessment" (mechanism currently unknown) of the X:A ratio.

In summation there are two major sex determining switch mechanisms (White, 1977): the dominant Y (or W, for the heterogametic female) and the genic balance system (X:A ratio). Triploids in the former system produce both males and females although sex ratios have been reported heavily skewed in one direction or the other. Triploids in the latter produce one sex and intersexes.

The soft-shell clam lends itself to a working hypothesis on sex determination because it is dioecious throughout its life, like the majority of pelecypods (Coe, 1943). The model that best accounts for our results is the X:autosome balance exemplified by *Drosophila.* The gonads of intersexes in *Drosophila* may show male or female differentiation (Baker and Belote, 1983); triploidy results in females and intersexes in an approximate 2:1 ratio because of the reduced viability of "male" intersexes. Of the 64 triploid clams about three-fourths were definite females. We expect that some intersexual clams would appear phenotypically as females, *i.e.*, with at least one or several oocytes, leading to the overestimate of this class. We further suggest that the triploid clams labeled as indeterminate are "female" intersexes and that the atypical triploid gametogenesis seen on 4/12 was that of a "male" intersex. We found 11 of these putative intersexuals and 4 other triploids with undeveloped sex characteristics.

ACKNOWLEDGMENTS

We thank Mindy Rice and Dianne Lincoln for their technical assistance in histological preparations. Dr. Edward C. Roosen-Runge provided a critical ear for histological interpretations. Drs. Gary H. Thorgaard and E. C. Roosen-Runge offered comments on the manuscript. We are also grateful to the anonymous reviewers for their constructive criticism. Financial support was provided by National Science Foundation Grant PCM-79-17029 to J.G.S. (while at the Univ. Maine, Orono) and H.H., and financial support for preparation of the manuscript was provided by NOAA Sea Grant # NA84AA-D-00011 to S.K.A.

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SHELL INORGANIC COMPOSITION AND ONSET OF SHELL MINERALIZATION DURING BIVALVE AND GASTROPOD EMBRYOGENESIS[a]

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ABSTRACT

Embryos of *Spisula solidissima* (Bivalvia); *Crepidula fornicata, C. convexa, Ilyanassa obsoleta* (Gastropoda: Prosobranchia); and *Dendronotus frondosus, Aeolidia papillosa,* and *Hermissenda crassicornis* (Gastropoda: Opisthobranchia) were reared in the laboratory and examined periodically to determine when shell mineralization began, as detected by birefringence under polarized light. Shell birefringence was detected at preveliger stages in all species. At onset of shell birefringence, samples were prepared for transmission electron microscopy to determine if the transitory shell field invagination (SFI) was still present. Contrary to previous reports for other molluscs, the present ultrastructural evidence indicates that at least for three species studied (*S. solidissima, A. papillosa,* and *H. crassicornis*), the SFI is still present during initial shell mineralization; thus for these three species the cells of the SFI may be involved in initial shell calcification. Electron cytochemical staining with pyroantimonate followed by electron probe microanalysis of thin sections indicated the presence of calcium within the lumen of the SFI in one species (*H. crassicornis*) fixed at onset of shell birefringence. These data suggest that the SFI may play an active role in initial molluscan shell formation. Analysis of veliger shell composition with scanning electron microprobe analysis indicated the variable presence of minor and trace amounts of Na, Mg, Al, P, S, Cl, and K in addition to large amounts of calcium. In two species (*C. fornicata* and *A. papillosa*) shell calcium carbonate was determined by X-ray diffraction analysis to be aragonitic.

INTRODUCTION

Molluscan shells consist of both organic matrix materials and inorganic crystalline substances (Wilbur and Saleuddin, 1983). Our current understanding of shell formation in bivalves and gastropods is based primarily on studies of adults or juveniles; very little is known about the formation and composition of molluscan shells prior to metamorphosis. In particular, only a few studies have dealt with the temporal and spatial localization of initial shell mineralization (Fretter and Pilkington, 1971; LaBarbera, 1974). Although embryonic and larval shells are often thought to be calcified (Robertson, 1971; Scheltema, 1971; Thiriot-Quiévreux, 1972; Boltovsky, 1974), the presence of calcium has generally been assumed rather than proven, and the developmental stage at which shell calcification begins within a species has rarely been

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Received 22 April 1985; accepted 19 January 1986.

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Abbreviations: SFI, shell field invagination; PPA, potassium pyroantimonate.

demonstration. Because the terms "embryonic shell" and "larval shell" are used differently by different workers, the timing of shell mineralization is often difficult to determine from published reports on the subject.

Fretter and Pilkington (1971) presented histochemical evidence for a lack of mineralization in the larval shells of several marine gastropod species. In non-marine gastropods and in marine gastropods with nurse eggs, calcification is said to take place only after metamorphosis to the juvenile stage (Kapur and Gibson, 1967; Bandel, 1982). Pechenik *et al.* (1984) and Eyster (1985), have recently utilized scanning electron microprobe analysis to demonstrate absence of significant calcification in larval and embryonic shells of the marine prosobranch gastropod *Cymatium parthenopeum* and the opisthobranch gastropod *Coryphella salmonacea* respectively. The degree to which timing of initial shell mineralization varies among taxa remains uncertain, however, partly because of terminological ambiguities and partly because of the false assumption that shell gross morphology can always be used to ascertain presence of shell calcification.

Prior to secretion of the first shell materials during embryogenesis, a portion of the embryonic dorsal ectoderm called the shell field temporarily invaginates, and then evaginates. As development proceeds, these shell field cells multiply and spread over much of the embryonic surface; it is this shell field epithelium that later develops folds or flaps and becomes the mantle (Kapur and Gibson, 1967; Timmermans, 1969; Demian and Yousif, 1973; Kniprath 1977; Shea and Morrill, 1982). The cells of the shell field invagination (SFI: = "shell gland") are generally believed to be responsible for initial shell secretion (*e.g.*, Fretter and Graham, 1962; Jablonski and Lutz, 1980). However, recent ultrastructural evidence from both bivalves and gastropods (Kniprath, 1977, 1980; Eyster, 1983, 1985; Eyster and Morse, 1984b) suggests that the cells of this invagination do not secrete the initial *organic* shell material; instead, the first organic shell material appears to be secreted by shell field cells lying immediately outside the invagination. The role of the invaginated cells in the secretion of early *inorganic* shell materials is unknown. Waller (1978) defined the SFI as the calcifying invagination of the ectoderm, but he did not provide any support for this statement. Moreover, several reports indicate that the SFI is absent at the time of first shell mineralization (Kapur and Gibson, 1967; Kniprath, 1977, 1979, 1981), in which case the invaginated cells can have no active role in initial calcification of the shell.

In the present work several bivalve and gastropod species were studied to determine the stage of development at which shell mineralization was initially detected and then to determine whether the shell field invagination was still present at the onset of shell mineralization. Electron cytochemical stains were utilized in an attempt to localize calcium in relation to the shell field cells at this stage of development. Also reported are the inorganic composition of embryonic and larval shells and the effects of several decalcifying agents.

MATERIALS AND METHODS

The following species were used in this study: the bivalve *Spisula solidissima,* the prosobranch gastropods *Ilyanassa obsoleta, Crepidula fornicata,* and *C. convexa,* and the opisthobranch gastropods *Dendronotus frondosus, Aeolidia papillosa,* and *Hermissenda crassicornis.* Adults of most species were collected from various locations in Massachusetts, except for *D. frondosus* (from Eastport, Maine) and *H. crassicornis* (from Californian adults; egg masses obtained from Dr. J. Harrigan, Marine Biological Laboratory, Woods Hole, Massachusetts). Because of the small size of the capsules of the opisthobranchs, embryos of those three species had to be examined and/or treated while still encapsulated.

"Embryo" is used here to refer to all prehatching developmental stages and "larva" refers to all free-swimming stages, as defined by Giese and Pearse (1974). Embryos and larvae of all gastropods were reared from eggs produced naturally in the laboratory. Eggs of the surf clam *Spisula solidissima* were obtained artificially via a gonad-slitting technique (Eyster and Morse, 1984a). All specimens were reared in clean glass dishes of natural seawater maintained under ambient (natural) photoperiods and temperatures.

Polarizing microscopy (pieces of polarizing film set at maximum extinction) was used to ascertain initiation of shell birefringence (see Figs. 1, 16 for examples of birefringent shell material). When the first weak birefringence was observed near the shell field, shell mineralization was said to have begun. That birefringence was due to shell mineralization and not to the shell organic matrix was corroborated by loss of shell birefringence during treatment with decalcifying agents.

Tetracycline was also used in an attempt to identify onset of shell mineralization; tetracycline binds to calcium and produces a fluorescent reaction product (Dey and Bolton, 1973). Embryos were reared in seawater containing tetracycline (50 mg/liter) and were examined periodically with fluorescence microscopy. Although a fluorescent reaction product (indicating shell calcification) was observed, it was not as easy to detect as was the initial birefringence. Therefore, polarizing microscopy rather than tetracycline/fluorescence microscopy was used routinely during this study.

Specimens examined periodically under polarized light were fixed for transmission electron microscopy (TEM) at onset of birefringence to determine if the shell field invagination (SFI) was still present. Standard TEM procedures (glutaraldehyde fixation, osmium-tetroxide post-fixation, ethanol dehydration) were utilized (Eyster, 1983). By necessity, all specimens were sectioned at random orientation.

In an attempt to localize calcium ultrastructurally at onset of birefringence, newly birefringent embryos were incubated either in 0.01–1.0 m*M* naphthaloylhydroxylamine (NHA) in glutaraldehyde (Zechmeister, 1979) or in a 2.5% solution of potassium pyroantimonate (PPA) and osmium tetroxide (Spicer *et al.*, 1968). For unknown reasons the NHA technique failed to produce an electron-dense reaction product even when the shell was known to be calcified, the embryos were incubated without prior glutaraldehyde prefixation, and the NHA concentration was increased two orders of magnitude over that suggested by Zechmeister (1979). The PPA solution was prepared by adding 1 g PPA (Pfaltz and Bauer, Inc.) in 20 ml distilled water; the mixture was heated to near boiling, cooled, reconstituted and mixed 1:1 with 2% osmium tetroxide in 0.3 *M* sucrose (Watabe and Blackwelder, 1980). Embryos and larvae were quickly rinsed with glutaraldehyde to remove sea salts that would precipitate the PPA. Specimens were incubated in the PPA solution for varying lengths of time (0.5–4 h), dehydrated in a graded ethanol series, and embedded in Spurr embedding medium. Sections were collected unsupported on copper grids and examined unstained on an RCA EMU 4 TEM at 50 kV or a Phillips 400 scanning transmission electron microscope (STEM) with an attached energy dispersive X-ray microanalyzer. Analyses of regions with reaction product and regions free of obvious reaction product were conducted for 100 seconds at 40 kV accelerating voltage.

The elemental (Na to Au) compositions of intact shells were determined with scanning electron microprobe analysis. Analyses were conducted with an AMR 1000A scanning electron microscope equipped with a Kevex detector and a Tracor Northern energy dispersive X-ray analyser. For all analyses the stub was set at an angle of 45° to the detector and an accelerating voltage of 20 kV was used. The electron beam was focused on six random locations on the outer shell surface for all specimens. By analyzing the wavelengths of the element-specific X-rays emitted, it was possible to identify the elements present in each of the probed regions for each shell (Goldstein *et al.*, 1981). The Bremsstrahlung background was subtracted and the total number of counts in each major elemental peak was integrated by computer.

Healthy, active individuals of *Spisula solidissima, Crepidula fornicata, C. convexa, Aeolidia papillosa,* and *Hermissenda crassicornis* were prepared for analysis of shell elemental composition. For several reasons, the specimens selected for elemental analysis had to be older (veligers) than the specimens used elsewhere in this study (trochophores). For microprobe analysis I wanted more shell material than was available at onset of shell mineralization. Also, for the opisthobranch gastropods, I had to use larvae (newly hatched) because the embryos were surrounded by tiny capsules, which could not be removed manually. Both air-dried and critical point dried specimens were prepared. For air-drying, the specimens were rinsed quickly in several changes of distilled water to remove sea salts. For critical point preparations, I exposed veligers to glutaraldehyde fixation, ethanol dehydration, and critical point drying with liquid carbon dioxide. Specimens prepared by both drying techniques were oriented and mounted in known orientation on standard aluminum SEM stubs and were desiccated over silica gel until time of analysis. Shells were not coated prior to analysis.

Additionally, shelled veligers of several opisthobranchs were incubated in 10 m*M* EGTA (ethylene-glycol-bis-N,N-tetraacetic acid) or in calcium-free seawater (CaFSW; calcium replaced with sodium; MBL Supply, Woods Hole, Massachusetts) to examine its effect on shell birefringence and elemental composition of the shell. The egg masses were kept in culture (natural seawater) until hatching was approached or reached. The masses were then cut into two or more pieces and transferred to fresh seawater, EGTA or CaFSW. Experimental details for *Dendronotus frondosus* and *Aeolidia papillosa* are given in the Results section. An egg mass of *Hermissenda crassicornis* was cultured in natural seawater until veligers naturally exited their capsules but had not yet begun to hatch from the gelatinous egg mass. The egg mass was divided into four pieces: C (control) SW-24h (controls in 0.22 μm Millipore filtered seawater for 24 hours), DW-24h (in distilled water, pH = 7.8, for 24 hours), and CaFSW-24h (in CaFSW for 24 hours). The C control shells were prepared immediately for elemental analysis by electron microprobe; shells from the other three groups were prepared for elemental analysis 24 hours later. For all groups, shells were selected and probed at random.

Specimens of *Crepidula fornicata* and *Aeolidia papillosa* were either treated with Feigl's solution (1% silver sulfate in 7.2% manganous sulfate monohydrate) or prepared for X-ray diffraction analysis to determine the crystal morph (calcite *versus* aragonite) in which shell calcium carbonate was deposited prior to hatching. For *C. fornicata,* prehatched veligers removed from capsules of one capsule cluster were used. For *A. papillosa* one egg mass in which embryos had just hatched from their small capsules but not from the egg mass was selected. For both species, the egg mass or capsules were cut open, and the shelled veligers removed, rinsed in several quick changes of distilled water, and transferred onto a glass strip cut from a standard glass microscope slide. As the water was drawn off and evaporated, the shells were pushed with a hair into a closely packed monolayer. No attempt was made to remove body tissues from the shells. Calcium carbonate standards were prepared by powdering aragonite and calcite separately with mortar and pestle, and adding acetone to the powder to produce slurries. These slurries were transferred to separate glass slides. Analyses of crystal morph were made using a Phillips X-ray diffractometer (Cu K_α radiation) with a scan speed of 1°/minute. Intensities of diffracted X-rays were examined from an angle of 20°–60° 2 θ.

RESULTS

The age at onset of shell mineralization for the seven species examined varied from one to fifteen days (after fertilization) depending on the species and the rearing temperature (Table I). However, in all species reported, mineralization of shell organic matrix began in preveliger individuals long before shell-like structures surrounded the young molluscs and long before metamorphosis.

At the time of initial shell mineralization, birefringence was best seen in all gastropod species when the subspherical embryos were oriented (as viewed through the microscope) with the newly secreted shell material lying along the edge of the embryo. When the shell material lay above or below the embryo, the birefringence was often

TABLE I

Initiation of shell mineralization, signalled by onset of shell birefringence. Age is measured from fertilization

Species	Age at onset	Temperature	Comments
Spisula solidissima	33 h	15°c	At 33 h, shell not visible with LM[a] and barely visible with SEM[b].
Crepidula fornicata	9 days	rm. temp. (~20°C)	Day 7: embryos ciliated, moving. Day 9: shell detectable with LM but onset of birefringence variable due in part to presence of runts.
Crepidula convexa	—	—	Youngest available embryos had feet and ciliated vela just forming, and already had strongly birefringent shells that shattered readily under pressure. Shells (140 × 200 μm) still smaller than embryos.
Ilyanassa obsoleta	6 days	rm. temp. (~20°C)	Day 5: first movement observed in some embryos. Day 6: shell birefringence observed in those embryos that had begun to move. Day 7: velar lobes distinct and slightly pigmented; no detected increase in strength of shell birefringence.
Dendronotus frondosus	8 days	8°C	Day 8: most but not all shells birefringent. Shell was capshaped, velum was forming, and statocysts were strongly birefringent (see Fig. 1).
Hermissenda crassicornis	4 days	15°–17°C	Shells of most embryos within egg mass became birefringent over 6 h period. 10 h before birefringence: no embryonic movement and no ciliary movement observed. 7 h before birefringence: cilia first observed.
Aeolidia papillosa	15 days	5°C	Day 13: embryos ciliated, began moving. 4 h after first birefringence: only 10% of shells in mass were birefringent. Day 16: all shells birefringent; shells not visible with LM. Day 18–19: shells were cup-shaped and surrounded posterior halves of embryos.

[a] LM = standard bright field light microscopy.
[b] SEM = scanning electron microscopy.

too weak [illegible] detected through the embryonic yolk. Initial shell birefringence was ti[illegible] of embryonic movement, perhaps partly because turning embryos [illegible] to be observed in advantageous orientations. Detection of shell bire[illegible] greatly improved in some species when pressure was applied to the [illegible] embryonic capsule until the shell separated from the underlying visceral [illegible] (Fig. 1).

Shell mineralization began when very little organic material was detectable with standard brightfield light microscopy, suggesting mineralization occurs progressively during rather than all at once after formation of the early organic shell. When viewed with shell material on edge at the time of first observed shell birefringence, *Hermissenda crassicornis* embryos had a birefringent region extending one-sixteenth their circumference; the shell was not yet obvious with standard light microscopy, indicating the shell was only a small cap at the time of initial mineralization.

Hermissenda crassicornis is the only species reared in this study that repeatedly had abnormal shell development (see also Hurst, 1967). Single egg masses contained embryos with semi-normal shells, many embryos with long and narrow birefringent shells, and some embryos that lacked birefringent shells but had birefringent statocysts.

The shells of newly hatched larvae of both *Aeolidia papillosa* and *Hermissenda crassicornis* did not collapse upon air drying, suggesting they were either well mineralized or sclerotized; loss of birefringence following treatment with decalcifying agents indicates the shells were mineralized and the lack of shell coloration may suggest the absence of significant sclerotization. As in other nudibranchs, the shells of *Dendronotus frondosus* remained transparent, birefringent, and cracked or shattered under pressure until discarded at metamorphosis. Newly birefringent shells of excapsulated *Crepidula fornicata* embryos deformed when allowed to air day, indicating that they were not heavily mineralized. The shells of excapsulated *C. fornicata* embryos several weeks in age lost their birefringence in 10 m*M* EGTA in about one hour (although at one hour the statocysts were still birefringent).

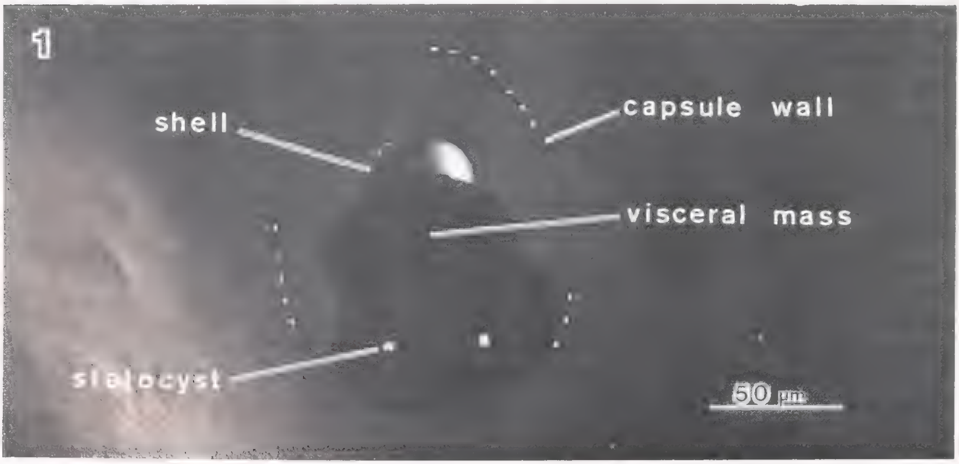


FIGURE 1. Dorsal view of encapsulated 8-day-old embryo of the opisthobranch gastropod *Dendronotus frondosus* observed with polarizing microscopy. To greatly increase visibility of the thin, newly mineralized shell, pressure was applied to the capsule, causing the cap-shaped shell to separate from the underlying visceral mass. Note that both the shell and the statocysts are birefringent.

The SFI at initial shell mineralization

Ultrastructural evidence from three of the above species (*Aeolidia papillosa, Spisula solidissima,* and *Hermissenda crassicornis*) fixed at onset of shell birefringence indicated that the shell field invagination (SFI) was still present when shell mineralization began. In sections through embryos of the opisthobranch *Aeolidia papillosa,* tiny patches (~1 × 2 μm) of microvilli were located between cells about 6–8 μm beneath the embryonic surface and the early organic shell (Figs. 2, 4). By sectioning younger embryos, (23 h before detected shell birefringence), in which the SFI lumen was larger and easier to detect, I was able to locate similar microvillous patches and to confirm that the patch of microvilli was the apparently wider, basal region of the small SFI lumen (Figs. 3, 5). The SFI lumen (in this and the other two species) opened beneath the center of the organic shell cap (Fig. 6); this fact enabled me to locate the very narrow SFI lumen by first locating the extraembryonic shell material. In sectioning through the edge of the SFI in these younger (prebirefringent) embryos, the upper region of the SFI lumen was seen as a narrow channel (Fig. 5). In *A. papillosa* embryos fixed at onset of shell birefringence, no channel was located (Fig. 4), presumably because of sectioning angle rather than absence of the SFI channel.

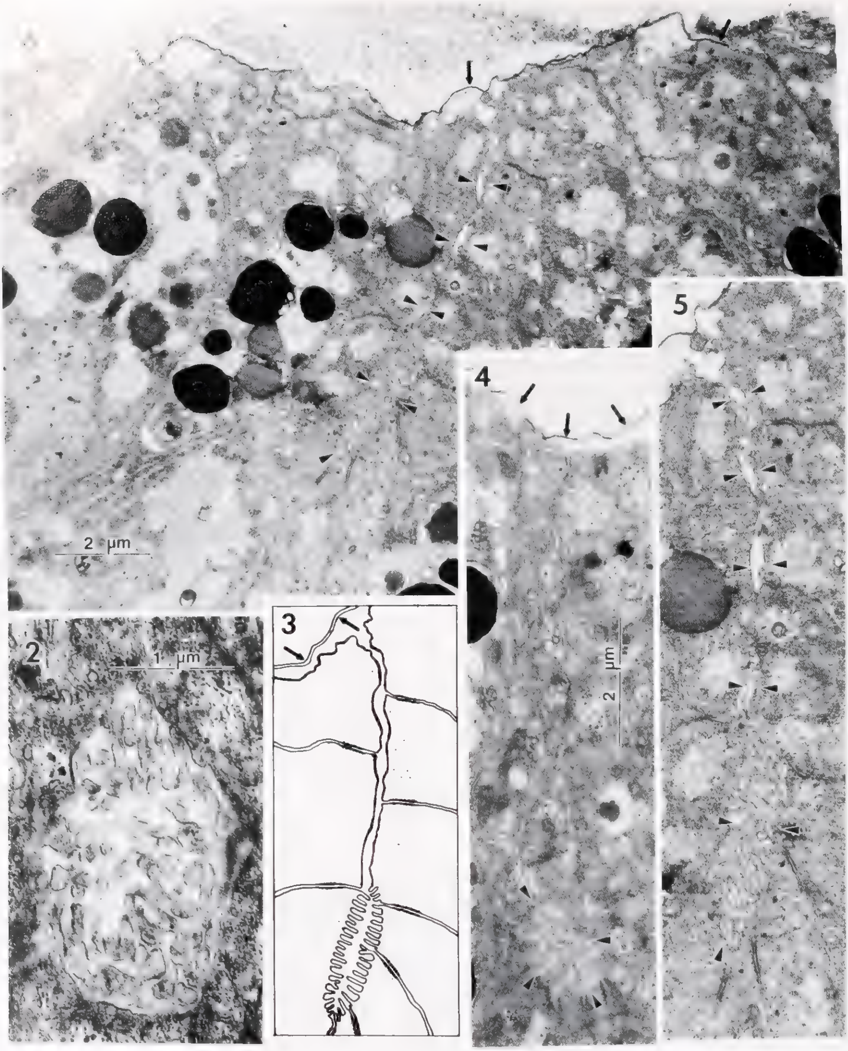
In the bivalve *Spisula solidissima,* an invagination was seen beneath the small amount of shell material present at first mineralization (Figs. 7, 8). This apparent SFI (the "inner invagination" of Eyster and Morse, 1984a) is narrow (0.5 μm or less wide) and similar to its appearance prior to shell mineralization. Unlike in *Aeolidia papillosa,* no distinct cluster of microvilli was observed lining the SFI lumen.

A narrow SFI channel was detected after onset of shell birefringence in a second opisthobranch, *Hermissenda crassicornis* (Fig. 9). The observed dimensions of this invagination were about 0.5–1.0 μm wide and 10 μm deep (Fig. 10). As in slightly younger embryos fixed prior to shell birefringence, this SFI lumen lay directly under the small cap of organic shell material, as diagrammed in Figure 9.

No SFIs were located at onset of shell mineralization in the other less well studied species. Because the embryos were too undifferentiated to be oriented for sectioning, and because the SFIs had apparently become so narrow, invaginated regions of the shell field were difficult to locate, even in the more well studied species described above.

Specimens of the three species in which an SFI was detected at onset of shell mineralization (*Aeolidia papillosa, Spisula solidissima,* and *Hermissenda crassicornis*) were incubated in an ultracytochemical stain for calcium at the time of initial shell birefringence. Because the quality of ultrastructural preservation is worse with osmium tetroxide—potassium pyroantimonate (PPA) than with standard glutaraldehyde—osmium tetroxide TEM preparations, it was more difficult to locate SFIs in these PPA incubated trochophores. The appearance of the SFIs located in two species are described below.

Larvae of *Spisula solidissima* fixed in osmium tetroxide—potassium pyroantimonate at the onset of shell birefringence showed electron-dense reaction product both inside and outside of shell field cells (Fig. 8) and other cells as well. Scattered granular reaction product was observed throughout the shell field cells of the inner invagination but not in obviously greater density than in non-shell field cells. Reaction product was particularly common over nuclei. In sections through the SFI lumen of one trochophore, a few scattered extracellular clusters of reaction product were seen within the lumen of the invagination; the largest cluster of reaction product was observed extracellularly, in the extrapallial fluid near the pore of the inner invagination (Fig. 8). Because the reaction product was calcium antimonate, as confirmed with



FIGURES 2–6. Sections through shell field cells of the opisthobranch gastropod *Aeolidia papillosa.*

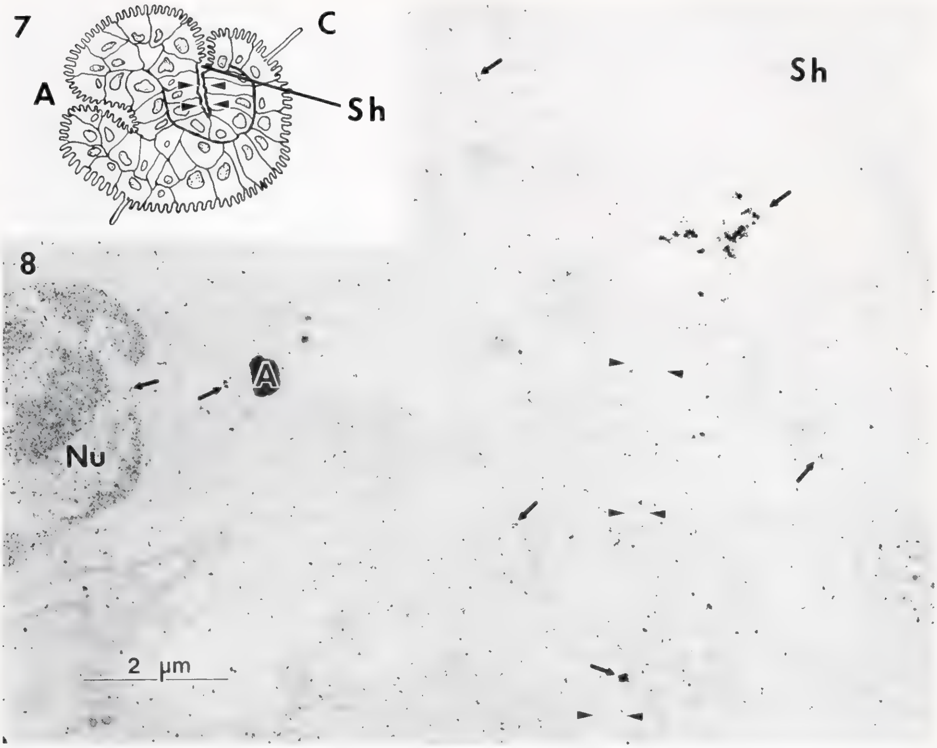
FIGURE 2. Microvillous region believed to be the basal (proximal) region of the SFI lumen detected after the onset of shell mineralization.

FIGURE 3. Schematic diagram of the proposed morphology of the shell field invagination of *A. papillosa* at the onset of shell mineralization. The basal region is lined with microvilli. The distal region is probably canal-like and may be microvillous. The pore of the SFI lumen opens beneath the shell, which at this stage of development consists of two electron-dense layers (arrows). Not to scale.

FIGURE 4. Micrograph showing the inconspicuous microvillous basal region (arrowheads) of the SFI lumen at onset of shell mineralization. The rest of the SFI lumen believed to be present (*i.e.*, a narrow canal connecting the microvillous basal region to the embryonic surface) was not detected. Part of the organic shell (arrows) is visible near the embryonic surface.

FIGURES 5 and 6. Micrographs showing the SFI lumen (arrowheads) at 23 h prior to observation of shell birefringence. The SFI lumen is actually much larger at this stage of development but is shown here in grazing section to demonstrate the similarity between the morphology of its basal region (Fig. 5) and that of the microvillous patch observed 23 h later, at onset of shell birefringence (Fig. 4).

FIGURE 6. Prior to mineralization the organic shell material (arrows) visible along the embryonic surface, isolates the pore of the SFI lumen from the external environment.



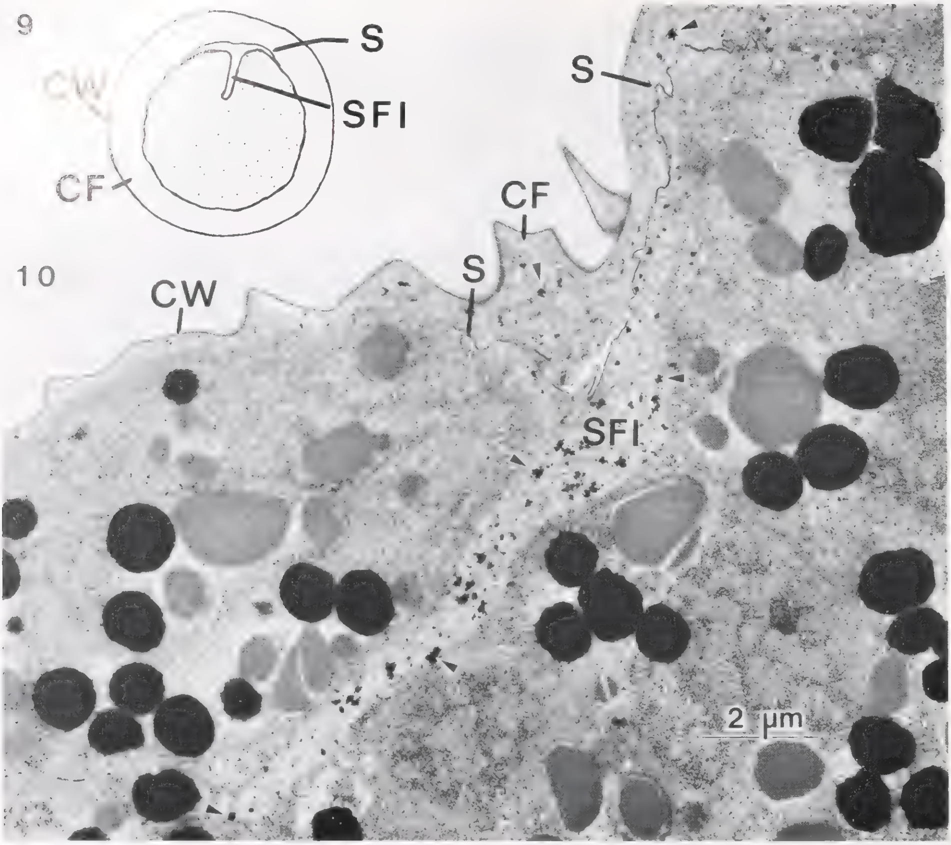
FIGURES 7 and 8. Sections through trochophores (33-h-old) of the bivalve *Spisula solidissima* at onset of shell mineralization. Both figures are in the same orientation. The SFI lumen (arrowheads) was still present.

FIGURE 7. Schematic diagram of section through ciliated (C) trochophore showing two invaginations, the archenteron (A) and the dorsal shell field invagination associated with formation of the early shell (Sh). The small amount of shell material present at this stage would lie at the tip of the pointer and is omitted for clarity. Not to scale.

FIGURE 8. Unstained section through a trochophore fixed in potassium pyroantimonate—osmium tetroxide to localize calcium at onset of shell mineralization. Electron-dense reaction product (arrows) was observed within the shell field cells, extracellularly within the narrow SFI lumen (arrowheads), and clustered at the junction of the SFI pore and the extrapallial space beneath the shell (Sh). Nu = nucleus. A = artifact.

transmission electron microprobe analysis, some calcium may be present in the lumen of the shell field invagination of *Spisula solidissima* during early shell formation.

In one embryo of the gastropod *Hermissenda crassicornis* incubated in PPA, an elongated region consistent with the location and morphology of the SFI was located (Figs, 9, 10). Within this region were large clusters of electron-dense reaction product, in addition to moderately electron-dense material resembling the 'albumen' of the capsular fluid found elsewhere between the embryo and the capsule wall. Within the shell field cells the reaction product was much finer grained (not clumped) than that observed extracellularly. Electron microprobe analysis of reaction product in the lumen of the SFI demonstrated the presence of calcium, as well as antimony and osmium from the fixative. Thus it appears that calcium may be present in the lumen of the shell field invagination of *H. crassicornis* at the onset of shell mineralization.



FIGURES 9 and 10. Sections through embryos (4-days-old) of the opisthobranch gastropod *Hermissenda crassicornis* at onset of shell mineralization.

FIGURE 9. Schematic diagram, not to scale, showing shell field invagination (SFI) and shell (S) in same orientation as in Figure 10. The embryo is surrounded by a capsule wall (CW) and the enclosed capsular fluid (CF). For clarity, the capsule is shown elevated from the embryo (as in life), rather than collapsed (as after fixation).

FIGURE 10. Transmission electron micrograph of an unstained section through an embryo, still inside its collapsed capsule (CW), fixed in potassium pyroantimonate—osmium tetroxide at the onset of shell birefringence. The shell field invagination (SFI) was observed beneath the center of the shell material (S). Electron-dense reaction product (arrowheads) containing calcium (and osmium and antimony from the fixative) was observed within the SFI lumen, suggesting calcium may be present in or transported to the SFI lumen during initial shell mineralization. CF = precipitated components of the capsular fluid.

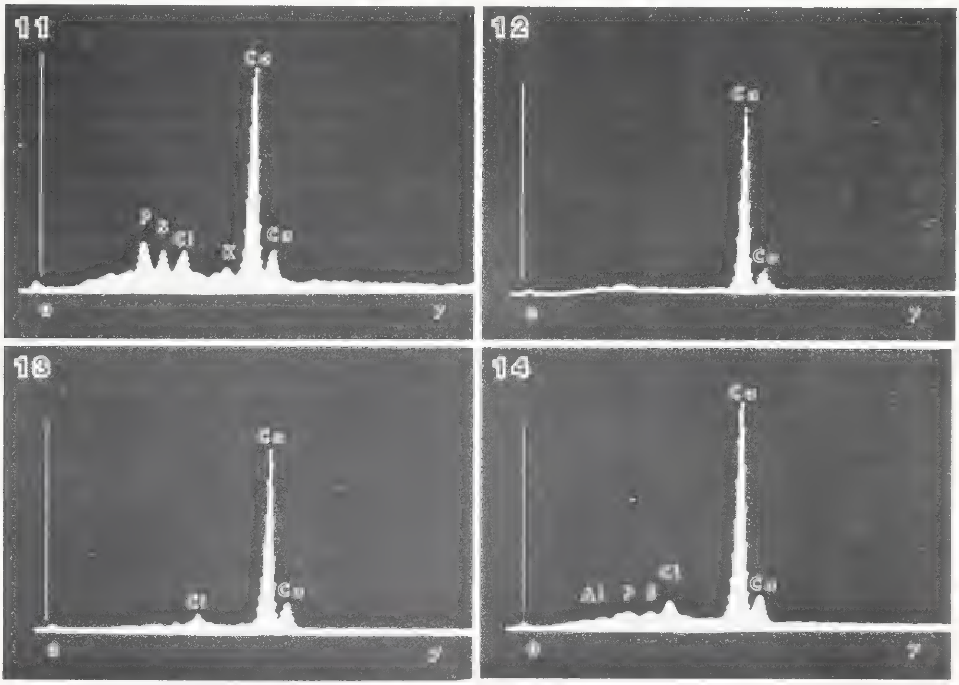
Ultrastructure of newly mineralized shell

At initiation of mineralization, the ultrastructure of the embryonic shell material remaining after standard aqueous TEM preparation appeared identical to its ultrastructure prior to mineralization. In both *Aeolidia papillosa* and *Hermissenda crassicornis* the newly mineralized organic matrix in section appeared as two similar electron-dense layers separated by an electron lucent zone, as diagrammed in Figure 3; it is unclear if this bilayer represents any or all of the periostracum. In newly mineralized shell of *Spisula solidissima* no obvious shell layers or compartments were observed.

The outer shell surfaces of several species were examined with SEM prior to microprobe analysis of shell elemental composition. For all nudibranch species examined, the larval shells lacked any sculptural pattern or obvious surface microstructure except for a series of raised lines near the aperture, as reported by Hurst (1967) (see Figs. 15, 17). SEM images of broken edges of air-dried *Aeolidia papillosa* larval shells did not reveal any crystalline microstructure, perhaps due to fracture mechanics (O'Neill, 1981). SEM of the shell surface of newly birefringent embryonic shells of *Crepidula fornicata* did not demonstrate any of the calcareous concretions described by Fretter and Pilkington (1971).

Shell composition and decalcification

Electron microprobe analysis of shell elemental composition (Na to Au) indicated that the shell mineral consisted almost entirely of calcium. Figures 11–13 show typical elemental spectra for probes of external shell surfaces. Untreated shells of most species generated large calcium peaks, with much smaller peaks for Na, Mg, Al, P, S, Cl, and K variably present (Table II). Of the non-calcium elements detected, chlorine was the most abundant and the most common. The concentration of each element, including calcium, was highly variable even from one location to another in a single shell, as demonstrated by standard deviation values (Table II). Based on probes near to and



FIGURES 11–14. Typical elemental spectra for shells prior to metamorphosis, demonstrating the presence of calcium for all species. X-ray energy in kiloelectron volts is given on the x-axes. The y-axes, representing the number of X-ray counts, are different for all graphs; to compare amounts of calcium detected see Table II.

FIGURE 11. *Crepidula fornicata.*

FIGURE 12. *Crepidula convexa.*

FIGURE 13. *Aeolidia papillosa.*

FIGURE 14. *Hermissenda crassicornis* reared for 24 h (after hatching) in calcium-free seawater.

TABLE II

Elemental composition (as determined with scanning electron microprobe analysis) of shells of five mollusc species prior to metamorphosis

Species/age, stage or treatment	Elements								N	Shell deformed on air drying
	Na	Mg	Al	P	S	Cl	K	Ca		
Spisula solidissima										
5 day old, 15°C	—	—	—	—	—	0–135	—	3092 ± 668	10	no
Crepidula fornicata										
10 day old[a], rm. temp.	t	t	—	152 ± 45	123 ± 10	294 ± 127	—	1532 ± 221	3	yes
10 day old[b], rm. temp.	—	—	—	182 ± 94	103 ± 33	129 ± 113	t	2313 ± 288	3	yes
Crepidula convexa										
prehatch	—	—	—	—	—	—	t[c]	5160 ± 2700	2	no
Aeolidia papillosa										
control (hatching)	—	—	—	t	—	198 ± 128	—	7571 ± 2075	12	no
into CaFSW in mass, 12d	t	t-180	—	—	144 ± 76	733 ± 216	—	5640 ± 705	6	no
Hermissenda crassicornis										
control (just hatched)	—	—	0–131	0–75	176 ± 64	273 ± 127	t-108	12756 ± 5788	6	no
SW-24h	—	—	66 ± 11	61 ± 4	112 ± 4	331 ± 30	t	12505 ± 2562	2	no
DW-24h	—	—	t	t	103 ± 23	242 ± 43	t	13107 ± 389	2	no
CaFSW-24h	—	—	121 ± 87	106 ± 56	135 ± 75	0–928	t	8853 ± 3385	8	yes

[a] Foot and operculum present, eyes starting to form, shell and statocysts birefringent.

[b] Further developed than [a].

[c] Trace Fe and Zn also detected.

Numbers represent mean ± S.D. N = number of probes. Trace (t) amounts of specific elements were not detected with all probes of one shell or of all shells of one species. Background readings ranged from 5 to 49 (mean ± S.D. = 27 ± 13).

distant from the shell aperture (Gastropoda) or the shell valve edges (Bivalvia), no differences in spatial distribution of the elements were correlated with newly mineralized *versus* older shell material.

Critical point dried shells of some larvae prepared for SEM/microprobe analysis had less calcium than air-dried shells of siblings. In fact, some critical point dried shells became quite wrinkled (Fig. 15), and revealed little or no calcium during electron microprobe analysis. These observations indicate that critical point drying procedures may partially or totally decalcify these lightly calcified samples and should not be used prior to mineral analyses.

The effect of decalcifying agents on shell birefringence and shell elemental composition varied with the decalcifying agent, the animal species, whether or not the animal was alive, and whether or not the animal was within some form of embryonic capsule and/or gelatinous mass. For example, shells of encapsulated *Dendronotus frondosus* veligers (removed from the egg mass) were still highly birefringent after 30 minutes incubation in 10 m*M* EGTA. However, outside of both the gelatinous mass and the egg capsules, the shells of live *D. frondosus* veligers decreased in birefringence within three minutes of incubation in the same solution, suggesting that the egg capsule is an effective barrier to the calcium chelator, EGTA. Also, shells of recently dead *D. frondosus* veligers decalcified more rapidly than did shells of live veligers; empty shells, newly discarded by metamorphosing individuals, decalcified even more rapidly. Shell decalcification was uneven or patchy, suggesting either variable thickness of a uniformly calcified shell or variable distribution of calcification. In shells of living veligers, decalcification was seen first at the aperture lip, perhaps because the new mineral solu-

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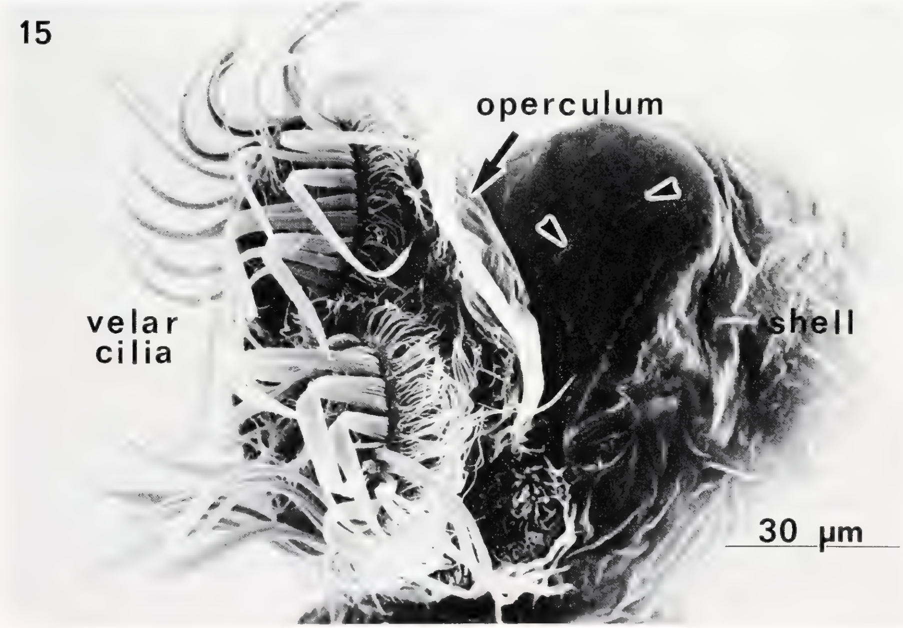


FIGURE 15. Scanning electron micrograph of larva of the opisthobranch gastropod *Aeolidia papillosa*, lying on its dorsal-lateral surface. Raised lines (arrowheads) are visible on the shell near the aperture. The shell is wrinkled because it decalcified during critical point drying procedures, which must be avoided prior to analysis of shell mineralization.

bilized [illegible] (see Akberali, 1980), because the shell was unprotected internally by living tissue or because this newly formed region is thinner. Shells of living veligers that were decalcified in EGTA and transferred back to control seawater were not remineralized by 48 hours, but were still a cohesive unit that was collectable by pipette. [illegible] from recently dead *D. frondosus* veligers left in static distilled water for two weeks or in seawater for two months decalcified but retained their shell shape. Although the organic shell remaining after decalcification retained its shape in static culture, it was flexible and readily collapsed when maneuvered.

For well-developed, encapsulated and enmassed *Aeolidia papillosa* veligers, incubation in CaFSW altered the elemental composition of the veliger shell as well as prolonged the duration of the prehatch period, and reduced by half the number of veligers that eventually hatched. By Day 12 of incubation all veligers maintained in normal seawater had hatched, swam upwards, became caught in the water surface, and died; all veligers subjected to CaFSW were also dead but their shells were opaque rather than transparent and, if hatched, were lying on the dish bottom rather than being stuck at the water surface. Unlike control shells, the opaque shells of treated individuals had detectable amounts of magnesium and sulfur (Table II). There was an obvious decrease in calcium levels in experimental shells.

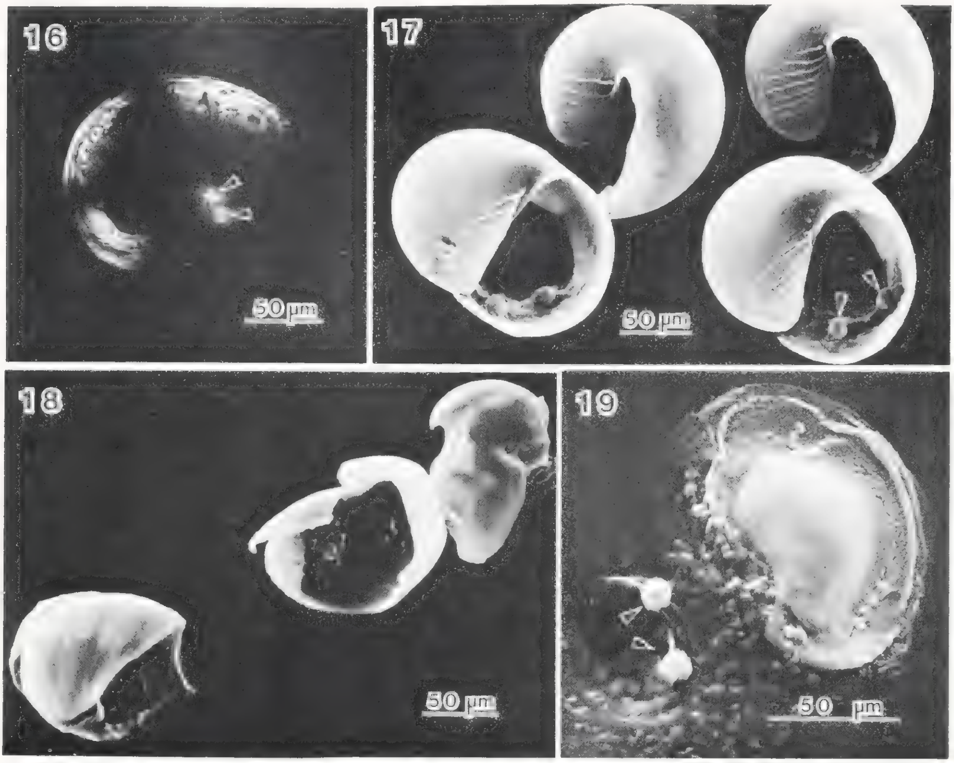
Incubation of newly hatched *Hermissenda crassicornis* veligers in CaFSW for 24 h also decreased shell calcium compared to that of controls. Control shells, even those trapped in the water surface, were birefringent (Fig. 16) and did not collapse upon air drying (Fig. 17). Inexplicably, entrapment in the water surface was associated with increased decalcification in CaFSW treatments. Shells of free-swimming treated larvae deformed visibly on air drying (Fig. 18), indicating partial decalcification. Other veligers in the CaFSW-24h treatment became trapped in the surface film, and although alive, completely lost shell birefringence; their shells, when allowed to air dry, totally collapsed (Fig. 19), indicating total shell decalcification. Their statocysts, surrounded by body tissues, were still birefringent after 24 hours.

Treatment of *Aeolidia papillosa* veligers with Feigl's solution resulted in the appearance of scattered clusters of black precipitate on the shell surface within five minutes, suggesting that the calcium carbonate of the shell is present as aragonite rather than calcite. X-ray diffraction patterns were obtained for premetamorphic shells of *Aeolidia papillosa* (Fig. 20) and *Crepidula fornicata*. These patterns resembled those obtained from X-ray diffraction of a powdered aragonite standard, demonstrating that the veliger shells for both species were aragonitic.

Discussion

Timing of initial shell mineralization

Gastropod species seem to differ in the timing of initial shell mineralization. In the present paper, shell mineralization began in all species at the trochophore stage of development shortly after the first organic shell material was secreted. In some other species, shell mineralization is said to begin later in development. For example, in some marine gastropods that feed on nurse eggs while encapsulated, shell mineralization is delayed (Bandel, 1975, 1982; Rivest, 1983). Teleplanic gastropod larvae may delay abundant shell calcification until after metamorphosis (Pechenik *et al.*, 1984), as an adaptation for long-distance dispersal (Scheltema, 1971). The freshwater gastropod, *Helisoma duryi*, is reported to delay shell calcification until hatching; even a few days after hatching, only occasional crystals of calcium carbonate were observed



FIGURES 16–19. Shells of newly hatched veligers of the opisthobranch *Hermissenda crassicornis.*

FIGURE 16. Right lateral view of control larva under polarized light, demonstrating birefringence of shell and statocysts (arrowheads). A large portion of the birefringent shell appears dark due to the maltese cross pattern.

FIGURES 17–19. Scanning electron micrographs of air-dried shells.

FIGURE 17. Shells of control larvae. The shells are calcified and show no signs of deformation on air drying. The only shell sculpturing present is a series of raised lines near the aperture. The two spherical structures (arrowheads) visible inside the shell are the statocysts.

FIGURES 18–19. Shells of larvae incubated in calcium-free seawater for 24 h. Shells of swimming larvae were birefringent but deformed during air drying due to partial decalcification (Fig. 18); shells of larvae stuck in the water surface were nonbirefringent, totally decalcified, and collapsed during air drying (Fig. 19). Statocysts (arrowheads) were still birefringent.

in the juvenile shell (Kapur and Gibson, 1967, 1968). In contrast, A. S. M. Saleuddin (pers. comm.) has observed prehatch shell calcification in *H. duryi. Coryphella salmonacea,* a nudibranch gastropod with benthic prejuvenile development, never calcifies its shell (Eyster, 1985); although an organic shell is formed it is not subsequently calcified. Since all nudibranch gastropods shed their shells at metamorphosis, lack of shell calcification by *C. salmonacea* may reflect adaptation to a non-planktonic existence.

According to Fretter and Pilkington (1971), the shells of *Crepidula fornicata* (and of several other prosobranch species) are fibrous, flexible, and do not significantly calcify until metamorphosis. In contrast, the shells of *C. fornicata* veligers observed in the present study were birefringent, shattered under pressure, exhibited prominent

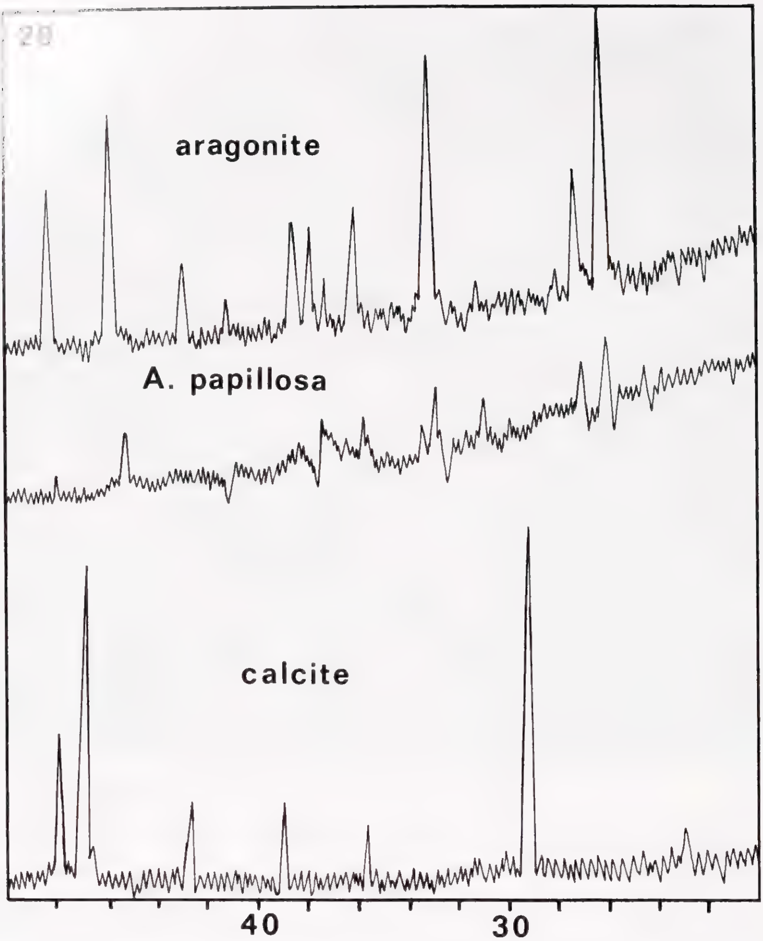


FIGURE 20. X-ray diffraction patterns for aragonite and calcite standards and for shells of recently hatched *Aeolidia papillosa*. Degrees (2θ) are given on the x-axis, for all three samples. Based on peak locations, the calcium carbonate of the shells of *A. papillosa* appears to be aragonitic.

calcium peaks with electron microprobe analysis, and produced peaks indicative of aragonite with X-ray diffraction analysis, all prior to metamorphosis. Clearly more work is required to determine if these apparent intraspecific differences in the timing of shell calcification reflect subtle taxonomic or geographic variation or perhaps differences in environmental conditions.

The early shell of *Crepidula fornicata* veligers was the least calcified of all species examined in the present study. The amount of calcium detected with the electron microprobe was lowest for this species and this is the only species in which untreated shells visibly deformed during air drying. In comparison, the shells of the congener *C. convexa* seemed to be more heavily calcified. The more lightly calcified shell in *C. fornicata* and the more heavily calcified shell in *C. convexa* probably reflect adaptations to presence of a larval stage in the former and the absence of a free-swimming stage in the latter.

Observations in the present study agree with previous reports (Cather, 1967; Ivester, 1972; Tompa, 1973) that mineralization of the shell of *Ilyanassa obsoleta* begins before hatching of the veliger larva. However, no TEM information is available on the morphology of the shell field at the onset of shell mineralization.

Onset of calcification among the Bivalva may be less variable than among the Gastropoda, but whether this is a real difference or is due to an insufficient number of studies on the subject remains to be seen. As pointed out by Waller (1981), the structure of the SFI and of the earliest formed shell is still one of the most poorly known aspects of bivalve larval morphology. The only study to detail the pattern of Prodissoconch I (= first shell) calcification was published in 1974 by LaBarbera. He reported that calcification began before the organic Prodissoconch I was completed, with mineralization beginning at the hinge and proceeding along the shell valve margins before progressing into the center of each valve.

It is not always possible to determine with light microscopy whether embryonic or larval shells are mineralized. For example, the transparent birefringent shells of larval *Hermissenda crassicornis* were mineralized (present study) while the transparent birefringent shells of larval *Cymatium parthenopeum* were not significantly calcified (Pechenik *et al.*, 1984). It is even more hazardous to guess whether shells depicted in the literature are (or were) mineralized. Belisle and Byrd (1980) published SEM micrographs of a *Thais haemastoma* developmental series. They did not mention whether the embryonic shell was calcified (it was not relevant to their study) but after looking at their Figures 32–33 the reader can probably safely conclude that the wrinkled shell is not calcified; however, the reader cannot tell whether the shell decalcified during fixation—critical point drying procedures or was not yet calcified.

It would be helpful if future reports on initial shell mineralization would describe how presence of calcium was demonstrated and describe the developmental stage of the mollusc, rather than only saying that shell mineralization "begins early" or referring to mineralization of the "embryonic shell" or the "larval shell." In the present work, initial shell mineralization was confirmed with a combination of polarizing microscopy, decalcifying agents, tetracycline incubation followed by fluorescence microscopy, electron cytochemical staining followed by transmission electron microprobe analysis, X-ray diffraction analysis, and scanning electron microprobe analysis; shell mineralization began in early trochophores (no mantles, no vela, no feet, no eyes) near the time of onset of ciliary activity. This is not to imply any causal relationship between onset of ciliary movement and shell calcification but to inform future workers of how developed the newly mineralized molluscs were in the present study.

Shell composition

Although the concentration and distribution of shell constituents have been examined in juvenile and adult molluscs (Rosenberg, 1980; Russell-Hunter *et al.*, 1981; Mackie and Flippance, 1983) little is known about the elemental composition of the shells of molluscs prior to metamorphosis. Electron microprobe analysis of the shells of newly hatched veligers of the opisthobranch gastropod *Coryphella verrucosa* revealed only calcium (Eyster, 1985). For veligers of the prosobranch gastropod *Cymatium parthenopeum* collected from the mid-Atlantic Ocean, Al, P, S, Cl, and low levels of Ca were reported from shell surface probes (Pechenik *et al.*, 1984).

The present study has demonstrated that large amounts of calcium and variable amounts of Na, Mg, Al, P, S, Cl, and K are detectable in the shells of some bivalve, prosobranch and opisthobranch molluscs long before metamorphosis. All of these elements have been previously reported in shells of postmetamorphic molluscs (Nicol, 1967; Carriker *et al.*, 1982; Roesijadi and Crecelius, 1984). Although chlorine was detected in most shells in the present study, it is not clear whether this element is an integral part of the shell or whether chlorine is an artifact from the sodium chloride of the seawater. The absence of a related sodium peak supports the suggestion that the chlorine is a true component of the shell, although sodium is near the detection limit of the equipment utilized. Aluminum has been previously reported from shell analyses of both premetamorphic (Pechenik *et al.*, 1984) and postmetamorphic molluscs (Nicol, 1967; Carriker *et al.*, 1982; Roesijadi and Crecelius, 1984). The facts that this element was not detected in all species in the present study and that probes of the tape between the aluminum stub and the shells did not give aluminum peaks support the supposition that the aluminum is part of or adsorbed to the shell. Sulfur previously has been reported present in shells of juvenile *Mytilus edulis* in the form of $SrSO_4$ and $BaSO_4$ (Travis, 1968), and in the insignificantly mineralized shell of *Cymatium parthenopeum*, possibly as a component of the sclerotized organic matrix (Pechenik *et al.*, 1984). However, in electron microprobe analyses of whole shells, it is impossible to ascertain whether the sulfur is an integral component of the organic matrix, the inorganic mineral, or both. As in shells of adult molluscs, there is no clear-cut distribution of these minor and trace elements within the early shell. It has been hypothesized (Carriker *et al.*, 1983) that the heterogeneous distribution of these elements results from incorporation of detritus into the shell during formation.

The present preliminary experiments involving exposure of veligers to calcium-free seawater (CaFSW) suggest that in the short term absence of environmental calcium some elemental substitution may occur. For example, magnesium was noted in all experimental *Aeolidia papillosa* shells but in no control shells. The alteration of shell elemental composition in even a few shells dried by critical point techniques is sufficient to make it clear that samples used in elemental analyses must instead be air dried, if at all possible. This is true even for lightly calcified shells that may deform or collapse during air drying. The shell decalcification observed in the present study during critical point drying must be attributable to one or more of the fixation/dehydration agents, but why it occurred in some preparations and not in others is unknown.

Role of the shell field invagination (SFI) in shell mineralization

Although for decades the transitory shell gland or invagination of the shell field of molluscs has been said to secrete the embryonic shell, no one has demonstrated that this structure actually secretes any of the organic or the inorganic shell components. Recent ultrastructural evidence argues against a role for the SFI in secreting the first *organic* shell materials (Kniprath, 1977, 1980; Eyster, 1983, 1985; Eyster and Morse, 1984b) and several studies have reported disappearance of the SFI prior to initial shell mineralization (Kapur and Gibson, 1967; Kniprath, 1977, 1980). Although current thinking leans toward support of the century old suggestion (see Kniprath, 1981 for review) that the shell field invaginates solely to bring in proximity the ring of cells that are capable of producing the first organic shell material, the present study provides the first ultrastructural evidence that the invagination of the shell field is still present during and may be involved in shell mineralization. Because shell mineralization may begin even before it is detectable with polarizing microscopy, the invagination may well be present during initial mineralization in more species than reported in this work. The absence of an SFI in sections of the less well studied species examined here (and in other species in the literature) does not prove that the SFI disappeared by the time shell mineralization began since all SFIs observed to date are extremely narrow and difficult to locate at this stage of development. More detailed studies are needed to determine whether the invagination is really absent at the onset of shell mineralization in any species, because if the SFI is absent, it can not play an active role in initial shell mineralization.

The present electron cytochemical localization of calcium in *Hermissenda crassicornis* embryos suggests that calcium might be transported through cells lining the shell field invagination, to the SFI lumen, and then to the inner surface of the newly formed shell organic matrix. If this pathway is correct, then the invaginated cells of the shell field would be actively involved in initial shell mineralization. However, the calcium localized in the SFI lumen in this study may have been transported there as an artifact of aqueous fixation. Also, the calcium may have entered the SFI lumen via the contiguous extrapallial fluid outside the lumen rather than through the cells of the SFI. Even if future investigations can demonstrate perhaps with ^{45}Ca, that calcium does exit the shell field cells during the invaginated stage, such evidence will not explain why an SFI develops. It is not obvious why a pocket of calcium-rich fluid in addition to the extrapallial fluid present at this and all later stages of shell formation would be beneficial during the initial stages of shell mineralization.

ACKNOWLEDGMENTS

I acknowledge Dr. Malcolm Hill (Earth Sciences Dept., Northeastern University) for assistance with X-ray diffraction analyses; Dr. Alan M. Kuzirian (N.I.H. Biophysics Lab, MBL, Woods Hole) for transmission electron microprobe analyses; Drs. Wm. J. Landis (Harvard Medical School and Children's Hospital) and Jan A. Pechenik (Tufts University) for useful discussions and comments on the manuscript; Len Sudenfield (M.I.T.) for scanning electron microprobe analyses; and Dr. Robert M. Woollacott (Harvard University) for generous access to his ultramicrotome.

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EFFECTS OF EYESTALK ABLATION ON LARVAL MOLTING RATES AND MORPHOLOGICAL DEVELOPMENT OF THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*

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ABSTRACT

Larvae of the lobster, *Homarus americanus,* displayed abbreviation of subsequent molt intervals in response to bilateral eyestalk ablation at the 2nd stage. The interval in which the ablation occurred (2nd stage) was occasionally shortened. However, the 3rd stage molt interval was always abbreviated. At the 4th stage the ablated larvae were larger than intact controls. Removal of the last pair of swimming legs, as a control for the trauma of eyestalk ablation, slightly decreased growth but had no effect on molt interval lengths. Eyestalk removal sometimes resulted in 4th stage larvae with characteristics intermediate between normal 3rd and 4th stages. Eyestalkless larvae that were reared communally and fed frozen *Artemia* formed significant numbers of these intermediates. Eyestalkless larvae reared individually and fed live *Artemia* nauplii rarely formed intermediates. These results imply that although eyestalks may be involved in the regulation of both the timing and growth increment of larval molting, they may not mediate the morphological aspects of lobster metamorphosis.

INTRODUCTION

Crustacean growth involves the interrelationship of two components. The first is the molt interval. Its regulation by molt-inhibiting hormone and ecdysteroids has been reviewed recently by Skinner (1985). The other component of growth is the molt increment, which is the increase in size that occurs from one stage to the next. Size increase occurs by absorption of water leading to an expansion of the new, soft exoskeleton and by slight stretching of the arthrodial membranes during intermolt (see Hartnoll, 1982, for review).

Development for many crustacean larvae consists of gradual morphological changes with successive molts until the first postlarval (juvenile) stage. In many instances, the gradual changes can be likened to those occurring in the hemimetabolous insects (Granger and Bollenbacher, 1981, for review). This process of gradual but pronounced changes through larval development can be considered a true metamorphosis (Passano, 1961). Usually, a standard number of stages is observed in laboratory rearing studies, each with its characteristic growth increment (Williamson, 1982). Variable numbers of stages, however, can also occur (Costlow, 1965; Williamson, 1982).

Marine crustacean larvae are usually tolerant of a wide range of temperature and salinity regimes. Suboptimal conditions can result in larvae that display extra intermediate stages (possessing characteristics of the normal preceding and subsequent stages) before completion of metamorphic development (Boyd and Johnson, 1963; Knowlton, 1974). Knowlton (1974) considered a variety of factors and reasoned that

Received 15 July 1985; accepted 8 January 1986.

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temperature and nutritional state were generally the most important in larval development.

Larval intermediate stages have also resulted from eyestalk ablation during zoeal development. The suggestion has been made that eyestalks are involved in the control of metamorphosis (Costlow, 1966b, 1968; Little, 1969; Charmantier *et al.*, 1985). Extra zoeal stages were observed after destalking the crabs *Sesarma reticulatum* (Costlow, 1966a) and *Rhithropanopeus harrisii* (Costlow, 1966b), and the shrimp *Palaemon macrodactylus* (Little, 1969). Intermediate stages in the lobster *Homarus americanus* were recently observed following eyestalk removal (Charmantier *et al.*, 1985) similar to those seen occasionally in normal laboratory-reared larvae (Templeman, 1936).

This paper presents additional data on the various effects of eyestalk ablation and rearing conditions on larval molting and development of *Homarus americanus*. We show that eyestalk ablation abbreviates subsequent larval molt intervals. We also provide evidence that suggests that eyestalks may not be involved in the mediation of morphological development of larval lobsters.

MATERIALS AND METHODS

Experimental animals and maintenance

American lobsters, *Homarus americanus*, used in these studies were from a variety of sources. Adult, egg-bearing females were kindly supplied by Dr. John Castell, Halifax Laboratory, Nova Scotia. Others were shipped from the Massachusetts State Lobster Hatchery, Martha's Vineyard, as gifts from John Hughes. Some of the females were cultured, and the larvae obtained at the Bodega Marine Laboratory by established techniques (Chang and Conklin, 1983; Hedgecock, 1983). Larvae were removed from the hatch system (18°C) within 6 h of hatching, placed into 4-liter beakers containing aerated, 1 μm-filtered, UV-irradiated seawater, and allowed to acclimate to the temperature at which they were subsequently raised (18–20°C, unless otherwise noted). For a single experiment, larvae from a single day's hatch were routinely used.

Larvae were individually transferred after acclimation to randomly assigned single compartments of plastic parts trays (Grainger's #A824). Compartments were filled with 50 ml of 1 μm-filtered, UV-irradiated seawater containing 12.5 units/ml of penicillin, 12.5 μg/ml of streptomycin, and 31.3 ng/ml of fungizone (antibiotic mix, Gibco). These concentrations are well within those used in other studies (Brick, 1974; Fisher and Nelson, 1977). As in those studies, the only observable effect of antibiotics on larvae was to prevent microbial infestations and thereby increase survival. *Artemia* nauplii, freshly hatched by the decapsulation method (Sorgeloos *et al.*, 1977), were fed daily to give a final concentration of 10/ml in each compartment. At this concentration, lobster larvae were fed to satiation as indicated by large numbers of *Artemia* nauplii remaining 24 h later. Equivalent concentrations of nauplii were given with each daily seawater (with antibiotics) change.

Molting experiments

In the first experiment, lobster larvae were transferred into plastic trays as outlined above. They were observed 3× daily for the appearance of newly molted 2nd stage zoea. This resulted in larvae that could be grouped within 8 h of their previous molt. Larvae were thus eyestalk-ablated at 24 ± 4 h after the molt. Larvae were randomly selected as intact controls and for eyestalk ablation or removal of the last pair of thoracopods (swimming legs). Larvae to be ablated were individually placed on a depression slide. The surrounding seawater was removed, and both eyestalks or thoracopods were severed at their bases using a pair of fine scissors (Vannas, Fine Science Tools). Larvae were allowed to remain on the slide for approximately 15 s for initial bleeding before placement into fresh seawater. Controls were placed on depression slides in [illegible] seawater for an equivalent amount of time. Larvae were observed 3× daily to determine the mean molting intervals from 2nd to 3rd (2nd stage molt interval) and 3rd to 4th (3rd stage molt interval) stages. For the purposes of this work, the hatching molt stage from prelarva to the 1st stage lobster was not considered as the 1st molt interval (Davis, 1964; Aiken, 1980). Survival of ablated larvae was typically 95% or better under these conditions. Statistical significance was determined by Student *t*-tests.

Morphological experiments

Sibling larvae from a single day's hatch were placed into compartments as stated previously. They were reared throughout this experiment at 22–23°C and observed for molting to 2nd stage every 2 h. At specified times of 1, 3, 6, 12, 18, 24, 30, 36, 42, or 48 h after molting, larvae were destalked or left intact to serve as controls. Larvae were observed 3×/day for determination of the 3rd stage molt interval. Morphological measurements of the larvae were taken after the 3rd molt. These measurements consisted of antenna length from the base, carapace length from the posterior base of the eyesocket to the anterior edge of the first abdominal segment, and length of the cheliped propopodite (hereafter referred to as chela length). All measurements were made with the aid of a dissecting microscope (Wild M5) equipped with an ocular micrometer. Statistical significance of morphological and molt interval differences between groups was tested by ANOVA and Scheffe tests.

A second experiment was designed to determine the effect of rearing conditions on the development and molting of larvae that were destalked at various times. Upon hatching, larvae from a single day's hatch from another female lobster were placed into a larval rearing kreisel at 18°C (Chang and Conklin, 1983). Larvae were initially stocked at a density of 500/40-liter system, fed frozen *Artemia* (San Francisco Bay Brand) *ad libitum*, and checked 3×/day for the appearance of newly molted 2nd stage zoea. Larvae were removed after molt to 2nd stage, and were either reared individually in compartments as before (with *Artemia* nauplii) or left in the communal rearing kreisels. At 18 ± 4 h and 90 ± 4 h after the molt to 2nd stage, a portion of the kreisel- and compartment-reared larvae were destalked and returned to their respective rearing systems. Larvae were checked several times daily to determine the numbers molting to intermediate stages at the molt to 4th stage. The experiment was terminated before any of the larvae had completed the molt to 5th stage.

RESULTS

Molting experiments

In seven of nine replicate experiments, the 2nd stage molt interval was shortened by eyestalk ablation. In a typical experiment, intact lobsters had an interval of 4.3 ± 0.2 days, whereas eyestalk-ablated larvae had an interval of 4.1 ± 0.1 days (n = 74, $P < 0.001$). In contrast to the occasional (78% of the experiments) abbreviation of the 2nd stage molt interval, eyestalk ablation (during early 2nd stage) always shortened the 3rd and subsequent stages (Table I). To determine whether the trauma of eyestalk removal itself was affecting subsequent molts, an additional group of 2nd stage larvae underwent removal of the last pair of thoracopods. The results in Table I indicate that removal of swimming legs had no effect on the subsequent 3rd stage molt interval.

TABLE I

Third stage molt intervals of eyestalk-ablated, leg-ablated, and intact control groups of larval lobsters

Treatment	3rd stage molt interval (days)		
	Mean	S.D.	n
Intact controls	6.2	0.5	28
Eye-ablated	5.4[a]	0.7	31
Leg-ablated	5.9[b]	0.6	22

[a] Denotes significance from the control group ($P < 0.01$, other experiments $P < 0.001$).

[b] Denotes significance from eyestalk-ablated group ($P < 0.05$) and lack of significance from controls ($P > 0.05$).

Morphological experiments

The most apparent effect of ablation at various times after the molt to 2nd stage was the appearance of larval intermediate stages in the 4th stage. Figures 1–4 are photographs of the different stages. Early-intermediate 4th stage larvae were defined by their close resemblance to normal 3rd stage larvae. They had smaller antennae, carapace, and chelae lengths and molted earlier than the other groups (Table II). The exopodites (swimming appendages) were not greatly reduced as they were in the late-intermediates and normal 4th stage larvae (see below). Dorsal spines were still present and not reduced on the abdomen. Neither the tail spine nor telson lateral spines were reduced. Telson setae were not apparent on the fringe region in the early-intermediates. Their behavior closely resembled that of normal 3rd stage larvae. They were consistently observed to lie in a curled position with the abdomen flexed under the cephalothorax.

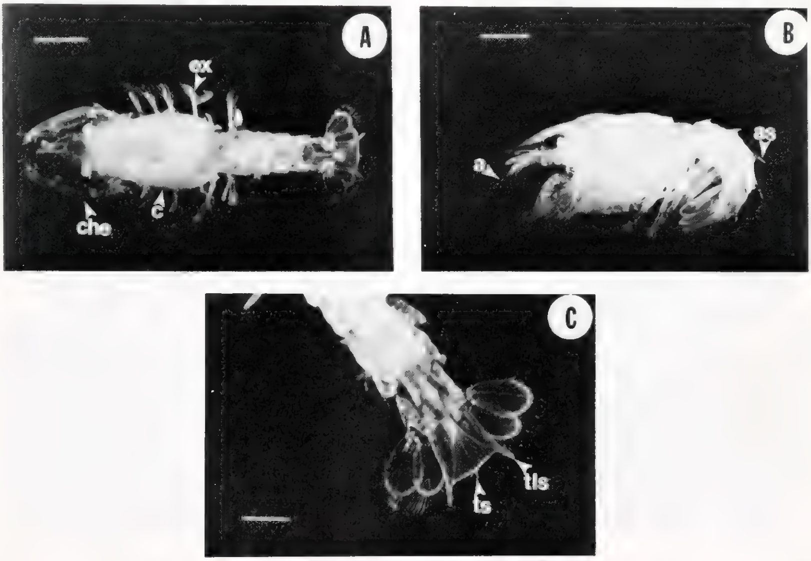

FIGURE 1. Early-intermediate larva at 4th stage. A) Dorsal view showing small chela (che), broad carapace (c), and exopodite (ex). Scale bar = 1 mm. B) Lateral view showing antenna (a) and abdominal spine (as). Scale bar = 1 mm. C) Dorsal view of tail showing telson spine (ts) and telson lateral spine (tls). Scale bar = 0.5 mm.

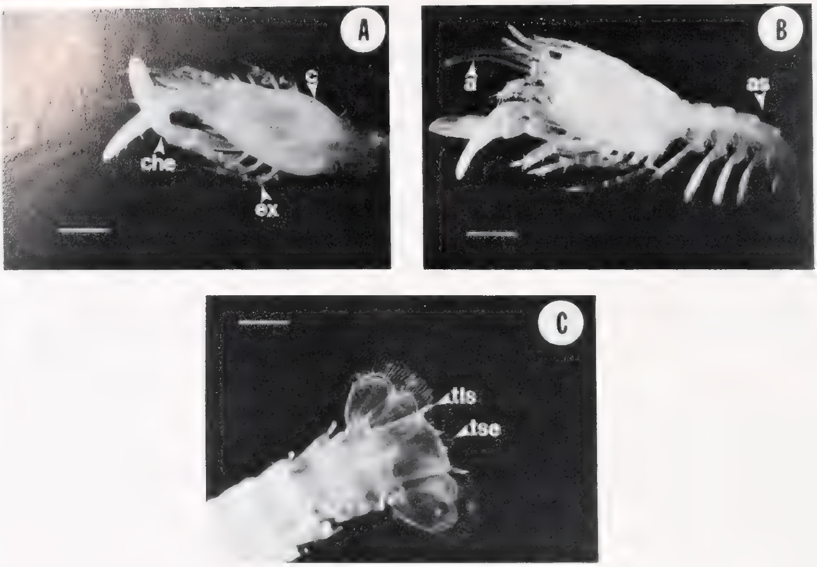

FIGURE 2. Late-intermediate larva at 4th molt. A) Dorsal view showing larger chela (che), normal-shaped carapace (c), and exopodite (ex). Scale bar = 1 mm. B) Lateral view indicating larger antenna (a) and reduced abdominal spine (as). Scale bar = 1 mm. C) Dorsal view of tail showing telson setae (tse) and reduced telson lateral spine (tls). Scale bar = 0.5 mm.

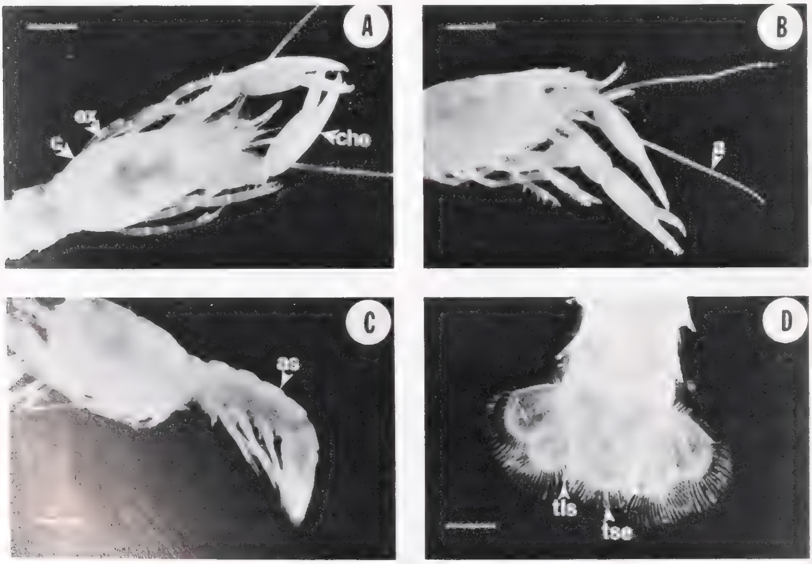

FIGURE 3. Normal 4th stage, eyestalk-ablated larvae. A) Dorsal view indicating chela (che), normal carapace (c), a lack of exopodite (ex). Scale bar = 1 mm. B) Lateral view of cephalothorax region showing normal length antenna (a). Scale bar = 1 mm. C) Lateral view of abdomen showing lack of abdominal spine (as). Scale bar = 1 mm. D) Dorsal view of tail region indicating normally reduced telson lateral spine (tls) and numerous telson setae (tse). Scale bar = 0.5 mm.

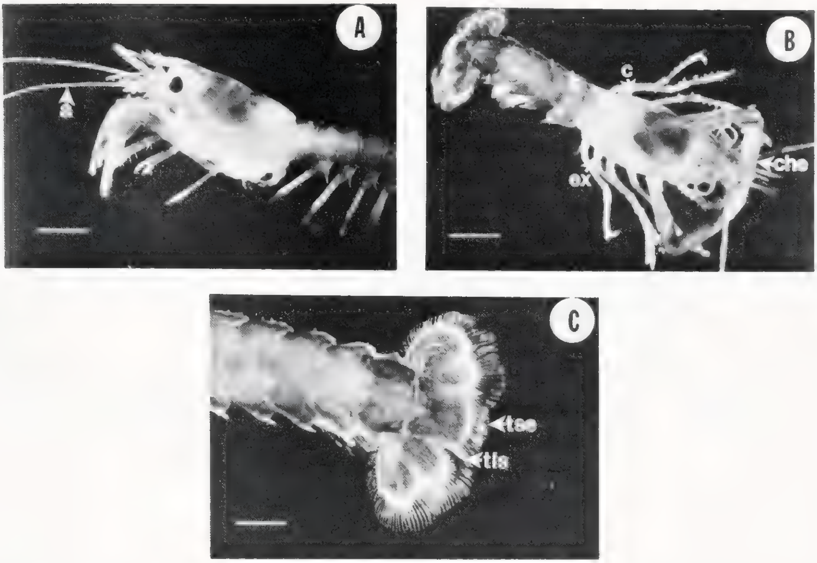

FIGURE 4. Normal 4th stage larvae. A) Lateral view showing antenna (a). Scale bar = 1 mm. B) Dorsal view indicating chela (che), carapace (c), and lack of exopodite (ex). Scale bar = 1 mm. C) Dorsal view of tail indicating reduced telson lateral spine (tls) and numerous telson setae (tse). Scale bar = 0.5 mm.

Normal 4th stage eyestalk-ablated larvae were significantly larger than controls and molted earlier to the 4th stage. Morphological development was equivalent to intact 4th stage larvae except for the size increases displayed by the eyestalk-ablated larvae.

Late-intermediate 4th stage larvae were morphologically more similar to normal (normally appearing) 4th stage larvae than were the early-intermediates. Antennae lengths were smaller than either the normal 4th stage ablated or intact control groups. Chelae lengths were larger than intact controls but smaller than normal 4th stage ablated larvae. Carapace lengths were larger than intact controls and equivalent to

TABLE II

Morphological and 3rd stage molt interval differences between early-intermediate (early-int., n = 102), late-intermediate (late-int., n = 29), normal-ablated (normal-abl., n = 32), and intact (control, n = 60) larval lobsters at 4th stage (means ± S.D.)

Treatment	Antenna length (mm)	Carapace length (mm)	Chela length (mm)	3rd stage molt interval (days)
Early-int.	2.6 ± 0.4	3.9 ± 0.2	2.7 ± 0.2	3.2 ± 0.5
Late-int.	a 4.3 ± 0.6	b 4.0 ± 0.1	a 3.5 ± 0.2	a 3.4 ± 0.5
	a	d	b	d
Normal-abl.	6.0 ± 0.5	4.1 ± 0.2	3.9 ± 0.2 c	3.8 ± 0.6 a
	b	a	a	c
Control	5.5 ± 0.5	3.7 ± 0.1	3.3 ± 0.1	4.8 ± 0.7

Statistical significance is indicated by the lines drawn between groups.

[a] Denotes significance at $P < 0.001$, [b] denotes significance at $P < 0.01$, [c] denotes significance at $P < 0.05$, [d] denotes lack of significance.

normal 4th stage ablated larvae (Table II). The exopodites were further reduced compared to the early-intermediates. The tail spine was much reduced or absent in these larvae. [illegible] lateral spines were more reduced and some fringe setae were apparent, [illegible] as numerous as those of normal 4th stage ablated larvae. Late-intermediate larvae molted significantly sooner than intact controls, however, not significantly sooner than normal 4th stage ablated larvae.

Intermediate stages appeared to a greater percentage the earlier the time of ablation of those larvae reared at 22°C (Table III). If eyestalks were removed up to 3 h after the molt to 2nd stage, 100% of the larvae molted to early-intermediates. From 6 to 18 h, about 80% became early-intermediates. There was a gradual decrease in the number of early-intermediates and a concomitant increase in late-intermediate and normal 4th stage ablated larvae when eyestalks were removed up to 48 h after the molt to 2nd stage. All of the intact controls molted to normal 4th stage larvae. Larvae from two additional females, however, did not form larval intermediate stages when eyestalk-ablated and reared at 22°C. These intermediate larval stages were rarely observed in many other experiments conducted at 18–20°C.

The effect of rearing conditions on the formation of larval intermediates was also investigated. Table IV shows the results of this experiment (conducted at 18°C). Almost all (97%) of the larvae that were ablated 12 h after molt to 2nd stage, returned to communal rearing kreisels, and fed frozen adult *Artemia,* developed into early-intermediates at the 4th stage. However, when larvae were ablated 90 h after molt to 2nd stage and returned to rearing kreisels, only 17.5% developed into early intermediates. Another 30% molted to late-intermediates and the rest molted to normal 4th stage ablated larvae. None of the kreisel-reared intact controls molted to intermediate stages. Upon molting to 2nd stage, an additional group of larvae from the same hatch were individually transferred from the kreisels to the compartmented trays and fed living *Artemia* nauplii. Some of these were ablated at either 12 or 90 h after the molt to 2nd stage. Ninety-one percent of the 12 h-ablated and 100% of the 90 h-ablated larvae molted to normal 4th stage larvae. As in the kreisels, none of the tray-reared intact controls molted to intermediate stages. Thus, depending upon the rearing conditions, either normal or intermediate larval forms could be induced in destalked larvae.

TABLE III

Morphological characteristics at the 4th stage of lobster larvae ablated at different times

Time of ablation[a] (h)	*n*	4th stage (%)		
		Early-intermediates	Late-intermediates	Normal 4th
1	12	12 (100)	0 (0)	0 (0)
3	14	14 (100)	0 (0)	0 (0)
6	9	6 (67)	2 (22)	1 (11)
12	15	13 (87)	1 (7)	1 (7)
18	16	14 (88)	1 (6)	1 (6)
24	16	8 (50)	3 (19)	5 (31)
30	18	11 (61)	4 (22)	3 (17)
36	17	5 (29)	4 (24)	8 (47)
42	18	6 (33)	7 (39)	5 (28)
48	18	3 (17)	7 (39)	8 (44)
Intact controls	60	0 (0)	0 (0)	60 (100)

[a] Refers to time of ablation following the molt to 2nd stage.

TABLE IV

Morphological characteristics at the 4th stage of eyestalk-ablated and control lobster larvae reared under different conditions

Time of ablation[a] (h)	% Mortality	*n*	4th stage (%)		
			Early-intermediates	Late-intermediates	Normal 4th
12, tray[b]	9.0	33	2 (6.1)	1 (3.0)	30 (90.9)
12, kreisel[c]	33.7	67	65 (97.0)	2 (3.0)	0 (0)
90, tray	16.0	21	0 (0)	0 (0)	21 (100)
90, kreisel	70.0	40	7 (17.5)	12 (30.0)	21 (52.5)
Control, tray	5.3	18	0 (0)	0 (0)	18 (100)
Control, kreisel	15.0	74	0 (0)	0 (0)	74 (100)

[a] Refers to eyestalk ablation at either 12 or 90 h after the molt to 2nd stage. Controls were intact larvae.
[b] Refers to rearing in compartmented trays and fed living *Artemia* nauplii.
[c] Refers to rearing in communal larval systems and fed frozen *Artemia* adults.

DISCUSSION

Molting rates

In general, eyestalk removal induces precocious molting in decapod crustaceans. This includes juvenile (Mauviot and Castell, 1976; Trider *et al.*, 1979; Chang and Bruce, 1980) and adult *Homarus americanus* (Mauviot and Castell, 1976). Our results support the observations of others of molt and growth rate enhancement by destalking lobster larvae (Rao *et al.*, 1973; Charmantier *et al.*, 1985). Removal of eyestalks early in the 2nd stage resulted in a slightly accelerated molt to 3rd stage. The molt intervals of the following stages were more significantly shortened. These results can be compared with work on other decapod larvae. Le Roux (1979) found that the first molt interval was unaffected following ablation in the anomuran *Pisidia longicornis*. The megalopal stage, however, was accelerated. The megalopal molt is accelerated in the crab *Callinectes sapidus* only if eyestalks are removed within 12 h of the previous ecdysis (Costlow, 1963). In a similar study of the crab *Sesarma reticulatum*, Costlow (1966a) reported that molting of magalopae and later stages was accelerated by ablation following the appearance of stalked eyes during zoeal development. Molting rates were unaffected by ablation in either zoeal or megalopal stages of the crab *Rhithropanopeus harrisii* (Costlow, 1966b). However, later work on *R. harrisii* larvae (Freeman and Costlow, 1980) identified an enhanced molting rate in megalopae only when eyestalks were removed within 12 h after the final zoeal molt. Those observations of molt acceleration by destalking of larval crustaceans support the original hypothesis of Brown and Cunningham (1939) on the control of molting by molt-inhibiting hormone.

Larval development

In addition to precocious molting, eyestalk removal can also lead to the development of intermediate stages. Our experiments were designed to examine the effects of rearing conditions on the induction of these intermediate stages in eyestalk-ablated lobster larvae. Eyestalk ablation of early and late 2nd stage larvae, that were reared in recirculating systems and fed frozen *Artemia* (know to be an inferior food source; Sorgeloos, 1980), resulted in numbers of 4th stage early-intermediates equivalent to those reported by Charmantier *et al.* (1985). Alternatively, larvae from the same hatch were removed, ablated at identical times, and then fed living *Artemia* nauplii (known to be a superior food source; Sorgeloos, 1980) in compartmented culture. These formed [illegible] intermediates when ablated early in 2nd stage and were 100% normal if ablated [illegible] the [illegible] premolt period. These results indicate that under suboptimal rearing conditions, eyestalks may not mediate the morphological aspects of metamorphosis.

Usually a finite number of larval stages are described for any given species (reviewed by Williamson, 1982). Stressful conditions can result in extra intermediate stages of natural or laboratory-reared crustacean larvae. However, larval development can be quite variable. Extra larval stages, or the retention of larval characteristics in postlarval stages can be induced by a variety of factors. Temperature (Boyd and Johnson, 1963; Knowlton, 1974), maternal influence (Templeman, 1936), diet (Broad, 1957; Knowlton, 1974; Sulkin, 1975, 1978), and starvation periods (McConaugha, 1982; Anger, 1984) have been identified or suggested as affecting morphological development. There are also a number of accounts of "larval intermediate stages" taken in plankton samples (Pike and Williamson, 1961; Nichols and Keney, 1963, as cited in Costlow, 1965; Knowlton, 1974, for review).

Larval intermediate stages can also result from eyestalk extirpation of decapod larvae. Little (1969) found extra larval stages in the shrimp *Palaemon macrodactylus* as a result of ablation during the 2nd–4th zoeal periods. Our first experiment on time of extirpation showed that nearly 100% of lobster larvae molted to early-intermediates when the operation was performed prior to 18 h of the 2nd stage. A greater percentage of larvae molted to late-intermediates or normal 4th stage larvae as the time of ablation approached 48 h. This period corresponds to early premolt as defined by changes in ecdysteroid titers (Snyder and Chang, 1986). Intermediate stage larvae also completed ecdysis earlier than the controls, although the difference was not statistically significant from late-intermediates. In a study by Le Roux (1979), *P. longicornis* retained some larval characteristics as juveniles when destalked on the 1st day of the 2nd zoeal stage. Costlow (1966b) observed that the crab *S. reticulatum* often went through a "2nd megalops" stage when larvae were destalked as early 2nd zoea. Critical periods for intermediate stage formation during zoeal development of *R. harrisii* (Costlow, 1966a) and *H. americanus* (Charmantier *et al.*, 1985) have also been described. The results reported here support the recurrent theme of a critical early zoeal period when suboptimal factors can result in subsequent intermediate stage formation. This observation is similar to that of Anger and Dawirs (1981) who described such a phenomenon with starvation. Survival of zoea to the next stage depended on their feeding from the beginning to at least 20% of the molt interval.

We observed that eyestalk-ablated larvae occasionally formed intermediates at the 4th stage. These observations imply that the stimulus for molting may take precedence over that for the completion of normal morphological development. This concept is supported by previous observations by others (Costlow, 1966a, b; Knowlton, 1974; McConaugha, 1982). However we were unable to repeat these results with larvae from two other female lobsters. Thus both parental and stress components may affect development (Templeman, 1936; Dawirs, 1983), possibly by the production of larvae that have insufficient energy reserves. Support for the importance of parental factors is the observation that broods from different females require varying lengths of time to complete metamorphosis in the laboratory (Boyd and Johnson, 1963; Costlow, 1965; Rabalais and Cameron, 1985).

The importance of food quality and nutritive reserves in larval development has been illustrated. Broad (1957) demonstrated that both food quality and quantity affected the molting rate and number of larval stages of two shrimp species. There is much evidence to support the idea that a diet of live *Artemia* nauplii or adults is capable of supporting normal early developmental processes in cultured marine animals (Fluchter, 1980; Sorgeloos, 1980). Normal growth is also supported by *Artemia* which have been preserved by rapid freezing in liquid nitrogen. Slow freezing (the process by which commercially available *Artemia* are frozen) results in a diet that does not support normal development, presumably due to the loss of nutrients (Fluchter, 1980). In our experiments, larvae were destalked after the molt to 2nd stage and fed either commercially frozen *Artemia* adults or freshly hatched nauplii. Virtually all of the ablated larvae that were fed nauplii developed normally to the 4th stage. Those fed commercially frozen adult *Artemia* exhibited delayed morphological development by molting to intermediate stages instead of normal 4th stage ablated larvae. These results extend earlier published observations that food quality may be a factor in the formation of larval lobster intermediate stages.

Eyestalk ablation is known to result in greatly increased respiration rates (Passano, 1961; McWhinnie and Kirchenberg, 1962). Alterations in amino acid (McWhinnie and Mohrherr, 1970), lipid (O'Connor and Gilbert, 1969), and sterol (Spaziani *et al.*, 1982) metabolism result from ablating adult decapods. Amino acid metabolism is also affected by eyestalk extirpation in *Callinectes sapidus* larvae (Tucker and Costlow, 1975). Coupled with the trauma that results from eyestalk ablation, and the inability to visualize prey, the above data support the idea that eyestalk-ablation alters normal life processes to an extent that, without high quality food, normal larval development is not possible.

This situation may be similar to that found in the holometabolous insect, the tobacco hornworm *Manduca sexta* (Safranek and Williams, 1984a, b). Malnourished larvae initiated metamorphosis only after reaching a critical weight. Supernumerary larval stages resulted if molting was initiated prior to the attainment of this weight. Analogous information on the control of metamorphic development in crustacean larvae is lacking.

ACKNOWLEDGMENTS

We thank N. Baum, M. Bruce, A. Hertz, and Dr. K. Nelson for many helpful discussions. We also thank Drs. D. Conklin and K. Nelson for critically reviewing the manuscript. This work was supported by a U. C. Davis Graduate Research Award (to M.J.S.), Sigma Xi (to M.J.S.), and NOAA, National Sea Grant College Program, Department of Commerce, under grant number NA80AA-D-00120, project number R/A-61, through the California Sea Grant College Program (to E.S.C.). The U. S. Government is authorized to reproduce and distribute copies for governmental purposes.

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EFFECTS OF SINUS GLAND EXTRACTS ON LARVAL MOLTING AND ECDYSTEROID TITERS OF THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*

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ABSTRACT

Eyestalk ablation of 2nd stage larvae of the lobster, *Homarus americanus,* resulted in an accelerated rise in whole larval ecdysteroid titers during the 3rd stage. The appearance of both the premolt ecdysteroid peak and the subsequent ecdysis were accelerated by eyestalk removal during the previous molt interval. Replacement therapy, using sinus gland (SG) extracts from juvenile lobsters injected into 3rd stage larvae (ablated during the 2nd stage), delayed the next molt. This delay was significantly longer than for larvae injected with non-sinus gland (NSG) eyestalk tissue extracts. Sinus gland extracts also decreased ecdysteroid titers of ablated larvae within 12 h. Basal levels were maintained in SG-injected larvae while control larvae reached the premolt peak. These results indicate that a molt-inhibitory mechanism similar to that of juvenile and adult decapod crustaceans may also exist in lobster larvae.

INTRODUCTION

Although much information has been gathered on juvenile and adult crustacean endocrinology, relatively little is known about hormonal regulation during the larval stages. Several studies have been concerned with the histological examination of eyestalk structures during larval development. The X-organ is present in all larval stages of the lobster *Homarus americanus,* but the sinus gland is not apparent until the 3rd stage (Pyle, 1943). Both structures are present in all stages of the shrimp *Palaemon macrodactylus* (Little, 1969), but in five species of the shrimp *Palaemonetes* the sinus gland is not apparent until the 5th stage and is probably not functional until the postlarval stages (Hubschman, 1963).

The role of ecdysteroids in larval insects has been heavily investigated (Koolman and Spindler, 1983, for review). Their role(s) in larval crustaceans is less well understood. McConaugha (1979) reared 1st and 3rd stages of the crab *Cancer anthonyi* in seawater containing 20-hydroxyecdysone. Accelerated molting in the presence of the hormone was not observed and a large percentage of the surviving larvae had abnormal morphological characteristics. Molting is accelerated in 4th stage *H. americanus* by injection of ecdysone or 20-hydroxyecdysone during early proecdysis (Rao *et al.,* 1973). Another indication of ecdysteroids acting as molting hormones in crustacean larvae is the demonstration of the characteristic cyclical pattern of larval lobster ecdysteroid titers coinciding with molting events during the first three stages (Chang and Bruce, 1981). The predominant hormone at the premolt titer peak was 20-hydroxyecdysone. In addition, a putative Y-organ has been identified in a larval crab (McConaugha, 1980).

Received 15 July 1985; accepted 8 January 1986.

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Abbreviations: sinus gland (SG), non-sinus gland eyestalk tissue (NSG), molt-inhibiting hormone (MIH).

Mechanisms that regulate larval crustacean molting cycles are poorly known relative to those of postlarval stages, although similar types of eyestalk ablation studies have been performed. Molting to the 1st crab stage is accelerated in megalopae of the blue crab, *Callinectes sapidus,* if both eyestalks are removed prior to a critical period (Costlow, 1963). Bilateral ablation during zoeal stages of the crabs *Sesarma reticulatum* (Costlow, 1966a) and *Pisidia longicornis* (Le Roux, 1979) has no effect on the duration of these stages. However, molting in the megalops stage is accelerated. American lobsters undergo precocious molting if ablated in the 4th (megalops) stage (Rao *et al.,* 1973). Zoeal molt cycle abbreviation was recently observed in lobsters by eyestalk extirpation (Charmantier *et al.,* 1985). In other studies, however, zoeal molting periods were unaffected by this treatment (Hubschman, 1963; Costlow, 1966b; Little, 1969). From earlier studies, Costlow (1968) inferred that the adult molt-controlling mechanism (molt-inhibiting hormone, MIH) was not operative in larvae. This idea was re-evaluated by Freeman and Costlow (1980). They proposed the existence of MIH in larvae of the crab *Rhithropanopeus harrisii,* based on observations of molt cycle abbreviation by eyestalk removal.

We report the results of experiments designed to investigate the presence of MIH-like activity in larvae of *Homarus americanus.* Previously, it was determined that eyestalk ablation of these larvae abbreviates subsequent molt intervals (Snyder and Chang, 1986). In the present study, ablated larvae were injected with sinus gland extracts possessing known MIH activity as assayed in juvenile lobsters (Bruce and Chang, 1984). Subsequent effects of these injections on molt intervals and whole larval ecdysteroid titers indicate that MIH may act to inhibit ecdysteroid production by the larval molting gland.

MATERIALS AND METHODS

Animal rearing and ablations

Larvae from American lobsters, *Homarus americanus,* were used in these studies. The sources of egg-bearing females were as stated previously (Snyder and Chang, 1986). Larvae were collected from a single day's hatch of a single female and stocked into individual plastic tray compartments. They were fed freshly hatched *Artemia* nauplii and reared at 18–20°C.

Ecdysteroid titers

Larvae were reared in compartments, and were either left intact or bilaterally eyestalk-ablated 24 ± 4 h after molting to 2nd stage (Snyder and Chang, 1986). In the first experiment, batches of 15 ablated and 15 control larvae were collected every 24 h. Groups of larvae were processed for radioimmunoassay (RIA) as follows. Larvae were blotted dry, weighed to the nearest 0.1 mg, and homogenized (20 strokes, Dounce homogenizer) in 1.25 ml of distilled water. The homogenate was precipitated with 3.75 ml of 100% ethanol (Gold Shield Chemical Co.) and centrifuged (5000 rpm, 10 min, Sorvall HS-4 rotor). The pellet was re-extracted with 1.0 ml of ethanol and centrifuged again. The combined supernatants were extracted twice with 0.75 ml of hexane. Samples of the aqueous layers were dried under vacuum and analyzed by RIA using the I-2 (17-week bleeding) antiserum of Horn *et al.* (1976) using the procedures detailed by Chang and O'Connor (1979). Samples were taken from hatching through the approximate midpoint of the 4th stage.

The second experiment involved larvae that were destalked 24 ± 1.5 h after molt to 2nd stage. Three samples, each consisting of 2 or 3 larvae, were taken every 12 h after eyestalks were removed until the end of the 3rd stage. Single homogenizations were performed in 0.25 ml of distilled water per 3 larvae. Methanol was added to a final concentration of 75%. Radioimmunoassays were performed after the centrifugation and hexane extraction steps. The antiserum used for these and all subsequent RIA determinations was the IB-4 serum, a gift from Dr. W. E. Bollenbacher, University of North Carolina. Statistical significance between ablated and control larvae was analyzed with Student *t*-tests. The extraction efficiency was checked by the addition of ^{3}H-ecdysone or ^{3}H-20-hydroxyecdysone (New England Nuclear) just prior to the homogenization steps. Mean efficiencies (for 4 determinations) were 85.3 ± 1.4% (ecdysone) and 86.7 ± 2.1% (20-hydroxyecdysone). Our data were not corrected for these extraction efficiencies.

Sinus gland injections

Sinus gland extracts from juvenile *H. americanus,* with molt intervals of 6–8 weeks, were prepared as described previously (Bruce and Chang, 1984). Molt staging of the juvenile donors was performed according to Aiken (1973). Only sinus glands from donors in the premolt stages were used. Eyestalks were removed and immediately chilled on ice. The sinus glands were dissected free from as much of the surrounding tissues as possible. Glands were pooled in a small drop of distilled water and homogenized in a micro-tissue grinder (Wheaton). The homogenate was lyophilized and stored at −75°C. Non-sinus gland extracts consisted of eyestalk tissue other than the sinus gland-X-organ complex. These tissues were estimated to be equivalent in size to the sinus glands utilized and were extracted in the same manner. Lyophilized extracts were reconstituted and used within 8 h.

Extracts were redissolved in sterile seawater, kept on ice for 10 min, and centrifuged for 2 minutes in a microcentrifuge (Beckman). The supernatant was reserved on ice for larval injections. Larvae were injected 24 ± 4 h (Exp. 1) or 24 ± 1.5 h (Exp. 2) after molt to the 3rd stage. In Exp. 1, both the intact controls and ablated larvae were injected with the extracts. Only ablated larvae were injected for Exp. 2. For the injections, the bevel of a #4 point, 33 gauge needle (Hamilton) was filed down to 25% of its initial length. Each injection contained 0.5 sinus gland (SG) or non-sinus gland (NSG) equivalent in 0.25 μl. Larvae were immobilized as for eyestalk ablation (Snyder and Chang, 1986), and injected once into the cephalothorax at a point just behind one of the fifth thoracopods. After allowance for initial blood clotting, larvae were returned to their rearing compartments. Lengths of the 3rd stage molt interval (and the 4th and 5th stage intervals for Exp. 1) were recorded every 8 h. For Exp. 2, the 3rd stage molt intervals were recorded every 2 h. Statistical comparisons were made with ANOVA and Scheffe analyses.

Injections and ecdysteroid titers

Larvae were destalked 24 ± 1.5 h after molt to 2nd stage as previously described (Snyder and Chang, 1986). The ablated larvae were randomly assigned to sinus gland-, non-sinus gland-, or non-injected groups. Injections were performed 24 ± 1.5 h after molt to 3rd stage as indicated previously. Triplicate groups of 2–3 larvae from each treatment were sampled by RIA every 6 h after injection of extracts until the molt to 4th stage. The mean values of each group at every time point were compared by Student *t*-tests.

RESULTS

Ecdysteroid titers

The total ecdysteroid titers for batch extracts of whole larval lobsters is shown in Figure 1. Titers exhibited the characteristic cycling with each molt interval. There was an initial postmolt basal level followed by a premolt increase to an ecdysteroid peak, and a final decrease signaling the onset of ecdysis. Peak hormone concentrations during the 1st through 3rd stage molt intervals for intact and ablated larvae were similar. The similarity was apparent whether the data were expressed on a per larva or per 10 mg wet weight basis. However, during the 3rd stage, the time of appearance of the peak titer was significantly earlier for the ablated larvae than for the intact controls. An earlier premolt rise was also observed for the ablated larvae in the beginning of the 4th stage.

Further characterization of ablated and control larval ecdysteroid titers was accomplished by taking more frequent, multiple samples. These results are shown in Figures 2 (2nd stage molt interval) and 3 (3rd stage molt interval). Eyestalk ablation resulted in a small, non-significant, increase in ecdysteroid levels during the 2nd stage. No differences between hormone levels were obvious though ablated larvae molted to the 3rd stage significantly earlier. Titer differences were more apparent during the 3rd stage. There was a significantly earlier rise in the hormone titers of ablated animals during the first day after ecdysis. In addition, the premolt ecdysteroid peak occurred significantly sooner in ablated larvae. These ablated larvae also displayed subsequent accelerated decreases of hormone concentrations culminating in earlier ecdyses.

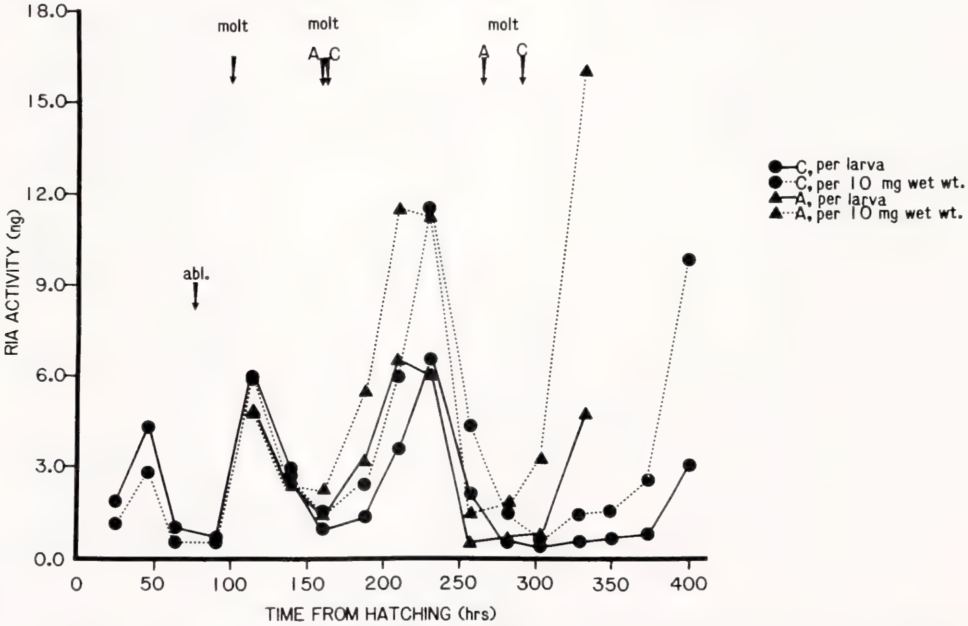

FIGURE 1. Ecdysteroid titers of eyestalk ablated (A) and control (C) lobster larvae from hatching through the midpoint of the 4th stage as determined by radioimmunoassay (RIA). Times of ablation (abl.) and molting are indicated by arrows.

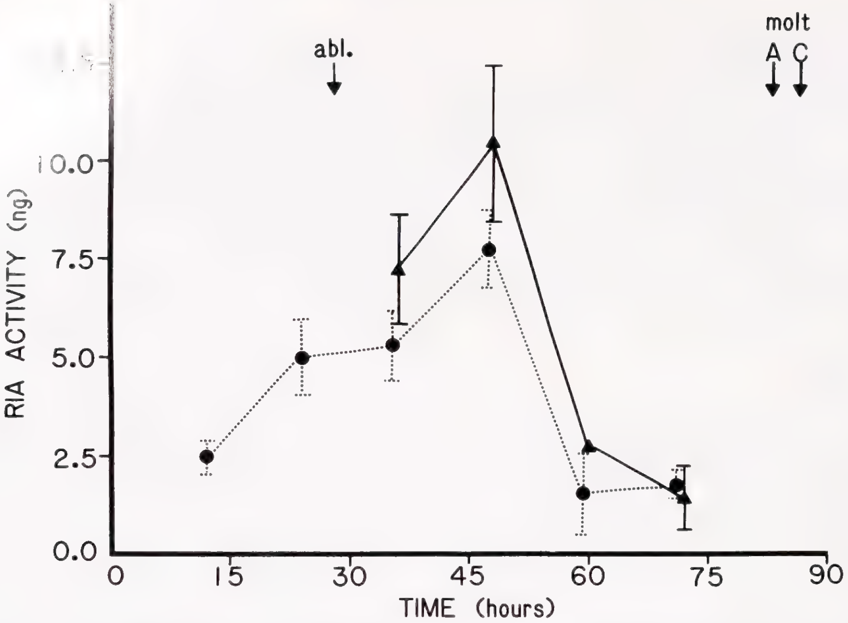

FIGURE 2. Ecdysteroid titers of eyestalk ablated (solid line) and intact control (dotted line) lobster larvae during the 2nd stage. Larvae were eyestalk-ablated (abl.) 24 ± 1.5 h after molt to 2nd stage. Symbols A and C refer to the time of molt to the 3rd stage for ablated and control larvae, respectively. RIA activity is reported on a per 10 mg wet weight basis for 3 samples of 2–3 larvae at each point. Bars are standard deviations of the 3 measurements.

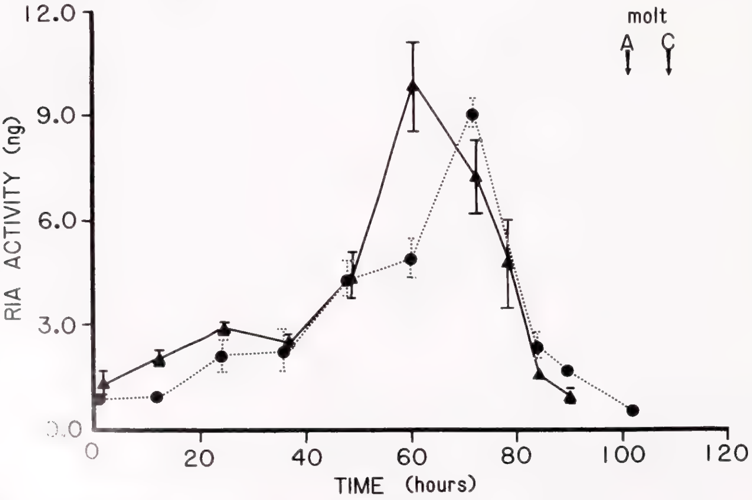

FIGURE 3. Ecdysteroid titers of eyestalk-ablated (solid line) and control (dotted line) lobster larvae during the 3rd stage. Larvae were eyestalk-ablated 24 ± 1.5 h after molt to 2nd stage. Symbols A and C refer to the times of molt to the 4th stage for ablated and control larvae, respectively. RIA activity is reported on a per 10 mg wet weight basis for 3 samples of 2–3 larvae at each point. Bars are standard deviations of the 3 measurements.

Injections and molting

Survival of extract-injected larvae was typically >75%. At 12–16 h after SG injection, blue coloration of the carapace was observed in ablated larvae, indicating that the injection had been successful. The color persisted for nearly 36 h, fading gradually. Non-SG injections had no such effect and the carapace remained white.

An indication of impending ecdysis could be made by the following observation. Within 12 h prior to a molt, larvae assumed an inverted position with the abdomen flexed dorsally towards the cephalothorax. This assisted in predicting subsequent molting events and hence, permitted closer observation.

Figure 4 shows the distribution of the 3rd stage larval molt intervals for the various treatments. It was observed that ablated larvae were significantly affected by the SG injections. There was a molt-delaying effect compared to both the ablated-non-injected ($P < 0.001$) and ablated-NSG-injected ($P < 0.05$) larvae. A portion of the molt interval delay in NSG-injected larvae could be related to the effects of the injection process alone. This is supported by observations that sterile seawater injections also delayed molt in some larvae (data not shown). Control, intact larvae were not significantly affected by SG injection. Further experiments concentrated on injection effects for ablated larvae only. The next two molt intervals of the injected larvae were followed. Table I shows that the injections (administered during the 3rd stage) had no effect on the subsequent 4th and 5th stage molt intervals.

To determine 3rd stage molt intervals more accurately, individual larvae were monitored every 2 h for molt to 4th stage. Molt intervals (in h) are given for this experiment in Table II. Sinus gland-injected larvae displayed molt interval lengths that were significantly longer than other groups ($P < 0.001$).

Injections and ecdysteroid titers

The above experiments demonstrated that injection of juvenile lobster sinus gland extracts increased the length of the molt interval. This phenomenon was investigated further by observing the ecdysteroid titers of SG-injected-, non-SG-injected-, ablated-non-injected-, and intact control larvae. Figure 5 shows the titers for all four groups.

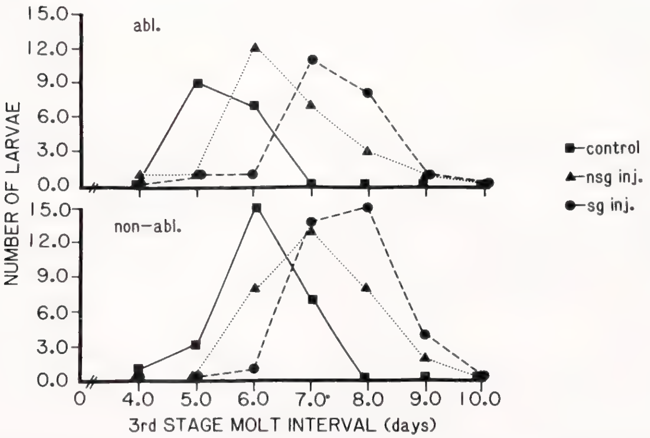

FIGURE 4. Distribution of lengths of 3rd stage molt intervals for ablated (abl.) and control (non-abl.) injected lobster larvae. Squares represent non-injected, triangles represent non-sinus gland (NSG) injected, and circles represent sinus gland (SG) injected larvae, respectively.

TABLE I

Length of 4th and 5th stage molt intervals of lobster larvae injected at 24 h after molt to 3rd stage

	4th Molt interval (days)			5th Molt interval (days)		
Treatment[a]	Mean	S.D.	n	Mean	S.D.	n
ES^-	10.5^b	1.1	15	10.8^b	1.4	15
ES^-, NSG	10.4^b	1.2	21	11.1^b	1.3	21
ES^-, SG	10.3^b	1.3	20	11.2^b	1.0	20
C	13.7	2.0	19	12.8	0.9	19
C, NSG	14.0	1.4	35	12.5	1.1	33
C, SG	14.1	1.7	32	12.8	1.4	32

[a] ES^- and C denote eyestalk-ablated and intact control larvae, respectively. SG and NSG denote larvae that were injected with either extracts of 0.5 sinus glands or with an equivalent amount of non-sinus gland eyestalk material, respectively.

[b] Denotes significance from control groups at $P < 0.001$; within group differences were not significant for eyestalk-ablated and control larvae.

Within 12 h of injection, SG extracts caused a decline in whole larval ecdysteroid levels. A low basal titer similar to normal postmolt levels was maintained until after the other groups had begun a late premolt titer decline. The SG-injected peak titer was slightly lower than that of the ablated-non-injected larvae and was delayed about 12 h from the NSG-injected group. These effects of sinus gland injections were both significant and reproducible. Although NSG injection seemed to cause a titer increase above the other groups in Figure 5, this effect was not statistically significant nor was it repeatable in other experiments. The molt intervals of SG-injected larvae were significantly longer than in any of the other groups.

DISCUSSION

Molting hormone titers

It has been shown previously that eyestalk ablation of 2nd stage larval lobsters accelerates molt intervals of subsequent stages (Snyder and Chang, 1986). Ecdysteroid

TABLE II

Length of the 3rd stage molt interval of lobster larvae injected at 24 h after molt to 3rd stage

	3rd Molt interval (h)		
Treatment[a]	Mean	S.D.	n
C	139.0	5.4	27
ES^-	$126.3^{b,d,e}$	4.6	15
ES^-, NSG	$137.3^{c,e}$	7.2	23
ES^-, SG	$151.7^{b,c,d}$	6.8	15

[a] Abbreviations for the various treatments are the same as for Table I.

[b] Denotes significance from non-ablated controls ($P < 0.001$).

[c] Denotes significance from ablated-non-injected group ($P < 0.001$).

[d] Denotes significance from ablated-NSG-injected group ($P < 0.001$).

[e] Denotes significance from ablated-SG-injected group ($P < 0.001$).

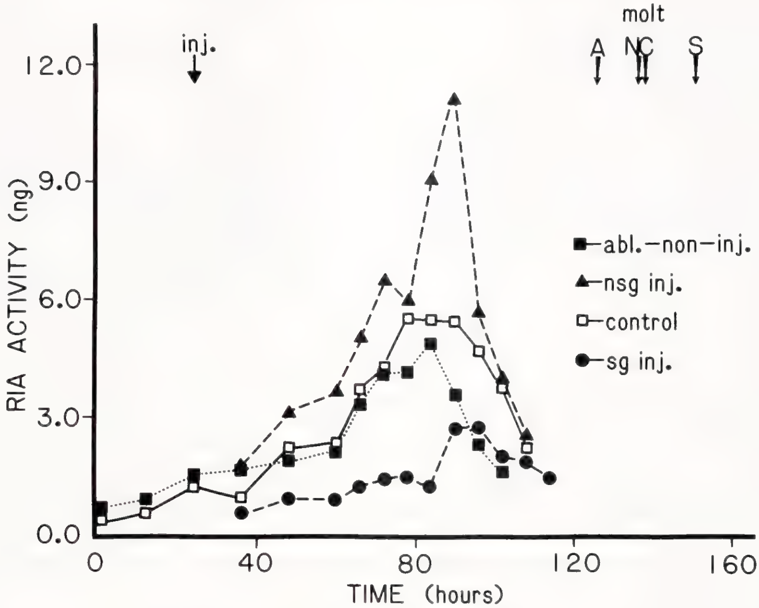

FIGURE 5. Ecdysteroid titers (S.D. bars removed for clarity) of ablated-injected, non-injected, and intact control lobster larvae during the 3rd stage. Eyestalk-ablated larvae were injected (inj.) with 0.5 sinus gland (SG) or non-sinus gland (NSG) equivalents at 24 ± 1.5 h after molt to 3rd stage. Symbols A, N, C, and S refer to the times of molt to 4th stage for ablated-non-injected (solid squares), ablated-NSG-injected (triangles), intact controls (open squares), and ablated-SG-injected larvae (circles), respectively. Larvae were ablated 24 ± 1.5 h after molt to 2nd stage.

titering of ablated 2nd stage larvae indicates a slight, insignificant increase during the premolt peak titer (Fig. 2). However, a statistically significant effect is observed in lobster larvae during the 3rd stage. A greater postmolt increase and an earlier premolt peak occur in ablated larvae during the 3rd stage. Concomitant with this effect is the observation of significantly abbreviated 3rd stage molt intervals. This is the first demonstration of the effect of ablation on the molting hormone titer in crustacean larvae. It is a very similar effect to that seen in juvenile lobsters that immediately display a premolt titer increase upon eyestalk ablation (Chang and Bruce, 1980).

These results support the idea of the presence of molt-inhibiting hormone (MIH) activity in decapod larvae, at least after the 3rd stage in *H. americanus*. First stage larvae do not survive ablation so it is difficult to ascertain the point of onset of MIH activity. The evidence suggests that the lack of an MIH-like factor(s) affects only the relative position of the premolt peak during the molt interval. These observations of the hormone titers of 1st and 2nd stage larvae and those by Chang and Bruce (1981) suggest that it may not be possible to further shorten the onset of the premolt ecdysteroid peak. The titer begins to rise in the typical premolt manner within 12 h of the previous molt in both stages.

Since ecdysteroid titers continue to cycle in eyestalk-ablated larvae, the question of the ultimate control of molting gland activity becomes apparent. In the absence of MIH, there must be an alternative mechanism for the control of ecdysteroid production. This concept has been reviewed by Skinner (1985). In addition, ablated juvenile lobsters that are well-fed continue to display cycling hormone titers and accelerated molting until they die (Chang, 1985). Results reported here with larval lobsters, as well as data on juvenile and adult crustaceans, suggest that ecdysteroid production may be controlled by an additional factor.

Injection effects on molting and edysteroid titers

Data reported here show that bilateral ablation of eyestalks accelerates the molt cycle in *H. americanus* larvae. Injection of juvenile lobster sinus gland (SG) extracts into these larvae at 3rd stage postmolt significantly delays the following molt (Fig. 5, Table II). Sinus gland tissue extracts delay molting significantly greater than non-sinus gland (NSG) extracts. This is the first demonstration of an effect of previously determined MIH-active substances (Bruce and Chang, 1984) on crustacean larvae. The slight molt-inhibition observed in NSG-injected compared to ablated-non-injected larvae may be due to an injury effect. Alternatively, the NSG tissues may have included small sections of the X-organ nerve tracts that contain MIH. In this instance, a very slight amount of MIH may have contributed to the molt delay.

Injections of extracts into intact larvae also were effective in delaying molt. There was not a specific SG effect however. This may have been due to the rapid degradation of the injected MIH factor(s) such that the molt delay could be attributed solely to an injury effect. An explanation for the lack of an enhanced inhibitory effect of SG relative to NSG injections is that endogenous hormone occupies most of the available MIH receptor sites. The injected hormone may then be degraded before the hormone receptors become available.

The results of extract injections on ecdysteroid titers support the data concerning the molt delay effect. Sinus gland injections depressed the ablated larval titers to basal levels within 12 h (Fig. 5). This effect was reproducible and more significant than observed in juveniles (Bruce and Chang, 1984). It is possible, therefore, that 3rd and 4th stage lobster larvae have a molt-inhibitory control mechanism(s) similar to that of juvenile and adult crustaceans. Pyle (1943) studied the X-organ-sinus gland system in larval lobsters using histological techniques. The X-organ was present in all larval stages, but the sinus gland was not found until the 3rd stage. Though Pyle found no evidence of neurosecretion in his study, the present results support the possibility that MIH is secreted from the eyestalk X-organ-SG complex during larval lobster development.

Sinus gland extract injections reduced ecdysteroid titers in the crab *Pachygrapsus crassipes* (Keller and O'Connor, 1982), and the ecdysone secretion of subsequently cultured Y-organs from ablated donor crayfish, *Orconectes limosus* (Gersch *et al.*, 1980). Sinus gland extracts administered *in vitro* reduced biosynthetic activity of cultured crayfish (Gersch *et al.*, 1977) and crab (Soumoff and O'Connor, 1982) Y-organs. All of these reports, in addition to our results on lobster larvae, suggest that the MIH factor(s) from the X-organ-SG acts upon the activity of ecdysteroid-synthesizing tissues to inhibit molting. The MIH factor(s) may also, however, act directly upon ecdysteroid target tissues (Freeman and Bartell, 1976; Freeman and Costlow, 1979).

In summary, it appears that regulation of larval decapod molting occurs in a similar manner to that found in juveniles and adults. Eyestalk removal results in both an earlier premolt ecdysteroid peak and the subsequent molt. Injection of juvenile lobster sinus gland extracts into destalked larvae delays the following molt. The SG injections also quickly diminish the whole larval ecdysteroid titer. The control of larval molting by an MIH-like mechanism which regulates ecdysteroid production is thus indicated. Future work will clarify the role(s) played by eyestalk neurosecretory centers in the regulation of larval molting processes. The rapid molt cycles of lobster larvae (about five days at 20°C for 3rd stage larvae) indicate that these animals are a good whole animal bioassay for the study of sinus gland peptides, in particular, MIH.

ACKNOWLEDGMENTS

We thank M. J. Bruce for help with eyestalk dissections. The gifts of anti-ecdysteroid antisera by Drs. W. E. Bollenbacher and J. D. O'Connor are gratefully acknowledged. We also thank Dr. K. Nelson for critically reviewing the manuscript. This work was supported by a U. C. Davis Graduate Research Award (to M.J.S.), Sigma Xi (to M.J.S.), and NOAA, National Sea Grant College Program, Department of Commerce, under grant number NA80AA-D-00120, project number R/A-61, through the California Sea Grant College Program (to E.S.C.). The U. S. Government is authorized to reproduce and distribute copies for governmental purposes.

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EXCESS POTASSIUM INDUCES LARVAL METAMORPHOSIS IN FOUR MARINE INVERTEBRATE SPECIES

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ABSTRACT

An increase in the concentration of K^+ in defined seawater medium induces settlement and metamorphosis in larvae of the marine molluscs *Phestilla sibogae, Haliotis rufescens,* and *Astraea undosa,* and in larvae of the marine annelid *Phragmatopoma californica.* The effect is dose-dependent, optimal at approximately double the normal concentration of K^+ in seawater, and specific for the K^+ ion. The ability of K^+ to directly influence cell membrane potential is proposed as an explanation for its broad effectiveness as a metamorphic inducer for larvae that recruit to different habitats. Depolarization of externally accessible, excitable cells thus is suggested to be a mechanism common to the induction of settlement and metamorphosis of a number of species. For *Phestilla* and *Haliotis,* the inductive effect of excess K^+ is additive with that of the substratum-derived inducers or analogs. The sensitivity of induction by K^+ to external tetraethylammonium (TEA, a K^+-channel blocker) reported previously for *Haliotis* (Baloun and Morse, 1984) is not present in *Phestilla* or *Phragmatopoma.* Results presented here indicate that the addition of excess K^+ may provide a widely useful technique for inducing metamorphosis, and for analyzing the mechanisms which govern this process, in other marine invertebrate larvae.

INTRODUCTION

Specific environmental stimuli are required for the initiation of metamorphosis by larvae of many marine invertebrates. In the plankton, these larvae typically develop competence to respond to metamorphic inducers, but do not proceed through metamorphosis in the absence of an appropriate stimulus. Often the stimulus is derived from a substratum capable of providing the newly settled juveniles with a source of nutrition and refuge; perception of this stimulus by larvae can involve chemical, tactile, or visual modalities (reviews by Crisp, 1974; and Hadfield, 1978). The sensory basis of induction suggests that the nervous system is involved in the initiation of metamorphosis (Hadfield, 1978; Burke, 1983a, b). In several species, substratum-associated morphogenetic chemical cues have been found to be neurotransmitter-mimetic substances (Morse, 1986); in these and other cases, exogenous neurotransmitters can elicit metamorphic responses similar to those induced by the natural cues, thus further implicating neuronal receptors in the initial processes (Bonar, 1976; Hadfield 1978; Morse *et al.,* 1979; Coon *et al.,* 1984; Morse and Morse, 1984a; Morse, 1986). Chemosensory receptors controlling metamorphosis of *Haliotis rufescens* (marine pro-

Received 13 May 1985; accepted 17 January 1986.

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sobranch gastropod) larvae in response to exogenous γ-aminobutyric acid (GABA) and GABA-mimetic compounds purified from the naturally recruiting host algae recently have been directly labelled and characterized (Trapido-Rosenthal and Morse, unpublished).

Signaling in many neuronal receptor systems has been found to involve depolarization of specialized cells in response to an appropriate stimulus (review by Aidley, 1978). We have suggested that the perception of inductive cues by larvae may rely on the stimulus-mediated depolarization of cells in a sensory/inductive pathway (Baloun and Morse, 1984). Consistent with this hypothesis, we previously observed that: (1) an increase in the concentration of K^+ in seawater is sufficient to induce settlement and metamorphosis of *Haliotis rufescens* larvae; (2) a decrease in external K^+ concentration inhibits the induction of metamorphosis in larvae of this species by GABA; and (3) sulfonyl isothiocyanostilbene, an anion channel blocker, also inhibits the larval response to GABA (Baloun and Morse, 1984). Since K^+ is capable of directly influencing cell membrane potential (Hodgkin and Horowicz, 1959), we further suggested that increasing external K^+ might be an effective technique for inducing metamorphosis in other marine invertebrate larvae. Evidence presented here demonstrates that increased potassium is effective as a morphogenetic inducer with larvae of several species of marine invertebrates. Larvae of the nudibranch *Phestilla sibogae* (Mollusca), the prosobranch *Astraea undosa* (Mollusca), and the polychaete *Phragmatopoma californica* (Annelida) respond to increased external K^+ in seawater with complete metamorphosis.

MATERIALS AND METHODS

Larval culture

Larvae were obtained from eggs of adult specimens of *Phestilla sibogae* maintained at the Kewalo Marine Laboratory, University of Hawaii. Fertilized egg masses were collected within hours of deposition, and maintaining in flowing seawater at 26.6 ± 0.4°C until larvae were hatched by gentle agitation of the egg cases at 5–6 days post-fertilization. Hatched larvae then were transferred into seawater with 60 ppm penicillin G and 50 ppm streptomycin sulfate in chambers aerated by airlift recirculation, where they developed to a stage of competence to respond to inducers of metamorphosis by 8 days post-fertilization (Bonar and Hadfield, 1974; Hadfield, 1977). An inductive extract of the natural recruiting substrate (the coral *Porites compressa;* Hadfield, 1977) was prepared by soaking broken tips of *P. compressa* for 20 h in 0.56 *M* NaCl, buffered to pH 8.3 with 10 m*M* tris(hydroxymethyl)aminomethane. The aqueous extract was passed sequentially through paper, 1.2 and 0.22 μm Millipore®, and Amicon® (nominal 10,000 MW exclusion) filters, and then adjusted to MBL seawater composition (see below) by the addition of K, Ca, and Mg salts and water before final dilution. The larval response to the extract is dose-dependent. For the coral extract used in experiments reported here, a dilution of 1 part of the MBL-adjusted extract to 20 parts standard MBL seawater induced approximately 30% of the larvae to metamorphose within 24 h.

Larvae of *Haliotis rufescens* were obtained by controlled fertilization, with gametes spawned by gravid adults after a brief exposure to dilute hydrogen peroxide (Morse *et al.*, 1977). Larvae were cultured in flowing 5 μm-filtered, ultraviolet-irradiated seawater at 15.0 ± 1.0°C; these uniformly developed to a stage of competence to respond to inducers of metamorphosis by 7 days post-fertilization (Morse *et al.*, 1980a). The amino acid GABA (γ-aminobutyric acid) effectively mimicks the action of the natural recruiting substrate (crustose red algae such as *Lithothamnium* and *Lithophyllum* spp.; Morse *et al.*, 1979, 1980a, b, c; Morse and Morse, 1984b). Conversely, the natural inducer purified from the recruiting algae mimicks the action of GABA at mammalian brain GABA receptors (Morse *et al.*, 1984; Morse, 1986). GABA at 4×10^{-7} *M* in MBL seawater provides a submaximal response that is sensitive to both facilitating and inhibitory conditions (Baloun and Morse, 1984).

Larvae of *Astraea undosa* were obtained by the mixing of gametes from adults stimulated to spawn by brief thermal shock in seawater at 24–25°C (an increase of 4–5°C above the broodstock ambient). These larvae were cultured at 18 ± 2°C in flowing 5 μm-filtered, ultraviolet-irradiated seawater. The natural inducer of metamorphosis for *A. undosa* larvae has not yet been precisely defined, although crustose red algae and GABA are effective (Markell and Morse, in prep.).

Larvae of *Phragmatopoma californica* were obtained by the mixing of gametes from adults stimulated to spawn by removal from their individual tubes (Jensen and Morse, 1984). Cultures of larvae, fed with the microalga *Dunaliella* spp., were maintained at 20 ± 1°C in stirred 5 μm-filtered, ultraviolet-irradiated seawater through development to metamorphic competence by 3 weeks post-fertilization. After development of metamorphic competence, the temperature of larval cultures was maintained at 17 ± 2°C. The natural recruiting substratum for this species is the anterior tube of the conspecific adult (Jensen and Morse, 1984). Clean preparations of inductive material were collected from tubes built in laboratory culture by adults provided with 0.45–0.50 mm glass beads (Glasperlen; B. Braun Melsungen AG Mfg., obtained from Van Waters and Rogers, Los Angeles, California; *cf.*, Jensen and Morse, 1984). Larvae of *H. rufescens, A. undosa,* and *P. californica* were cultured and used in experiments at the University of California, Santa Barbara.

Artificial seawater

Defined seawater medium based on the Woods Hole Marine Biological Laboratory (MBL) recipe (Cavanaugh, 1956) was used for all control assays, and was modified in experimental media by the addition of chloride salts to increase the concentrations of selected cations. The salt and ion concentrations of the MBL medium were summarized previously for reference (Baloun and Morse, 1984). The final pH of media ranged from 7.8 to 8.1 without adjustment. Prior to use, the antibiotics penicillin and streptomycin were added to the media, at concentrations of 50 and 60 ppm respectively for media used with *P. sibogae,* and at a concentration of 150 ppm each for media used with *H. rufescens* or *A. undosa.* No antibiotics were included in assays with *P. californica.*

Sigma and Mallinckrodt Chemical analytical reagent grade salts were used in the construction of artificial seawater media, with the exception of the highly hygroscopic salt $MgCl_2 \cdot H_2O$, which was purchased as a 4.9 *M* stock solution from Sigma Chemical Company. Tetraethylammonium chloride (TEA), a potassium blocker, was obtained from Eastman Kodak Company. Antibiotics were purchased from Sigma Chemical Company.

Assays of induction

The induction of metamorphosis of *P. sibogae* larvae was determined by the percentage of 11 or 12 day old competent larvae that discarded their shells and elongated into the characteristic juvenile form (Hadfield, 1978) after 1–3 days of exposure to a test medium. Duplicate or triplicate assays using 20 larvae in 20 or 25 ml of artificial seawater medium were carried out in lidded 40 ml Stender dishes at approximately 2[illegible]°C.

The induction of plantigrade attachment of *H. rufescens* and *A. undosa,* an accurate indicator of metamorphic commitment (Morse *et al.,* 1979, 1980b; Markell and Morse, in prep.), was quantitated as the percentage of larvae firmly attached by the foot, as a function of time of exposure. Approximately 200 to 300 competent larvae were tested in each glass vial (2.4 cm diam.) with 10 ml of artificial seawater medium; test vials of larvae were incubated in duplicate at 15.0 ± 1.0°C for *H. rufescens,* and at 18 ± 2°C for *A. undosa.* Completion of metamorphosis was verified by loss of the velar swimming organ and the initiation of adult shell growth.

The induction of metamorphosis of *P. californica* larvae was measured as the percentage of larvae that dropped provisional setae and rotated the anterior tentacles forward (Jensen and Morse, 1984). The induction of metamorphosis was assayed as a function of cumulative time of exposure for five larvae in each vial (2.4 cm diam.) with 10 ml of medium. Completion of metamorphosis was confirmed by the development of the caudal tail. Data for all species are presented as the mean percentage of larvae responding, determined from 2 to 4 replicates from single representative experiments with standard deviation (S.D.) as indicated.

RESULTS

Larvae of three species of gastropod mollusc and one species of polychaete annelid metamorphosed in response to increased external K^+ in seawater, in the absence of any other source of inductive stimulation. The response, measured as percent attachment or metamorphosis, was dose-dependent and yielded an approximately bell-shaped curve of effect as a function of concentration. At the lower limit, the response declined as the concentration of K^+ approached 0 m*M* excess, the non-inductive MBL seawater condition. The upper limit of the response was imposed by the onset of paralytic effects of high doses of excess K^+. However, within the effective tolerated range, the larvae remained healthy and active, and when induced, underwent complete metamorphosis.

Larvae of *Phestilla sibogae* showed an optimal response to increased external K^+ continuously provided at 20 m*M* excess (Fig. 1). By 72 h exposure to this concentration, 90% of the larvae metamorphosed into elongated shell-less juveniles. Attachment behavior, preceding the actual discarding of the shell, was not an unambiguous indicator of induction for *Phestilla;* thus we used completion of metamorphosis rather than initiation of the response as the measured parameter.

Larvae of *H. rufescens* and *A. undosa* displayed a rapid behavioral change in the presence of inducer, which provided a useful index of the induction of metamorphosis, and allowed quantitation of the initiation of the response. The attachment response, followed by complete metamorphosis, was optimally induced in *H. rufescens* larvae by about 10 m*M* excess K^+ (Fig. 2). Similarly, attachment and subsequent metamorphosis of *A. undosa* larvae occurred optimally in response to approximately 10 m*M* K^+ excess (Fig. 3).

Larvae of *Phragmatopoma californica* also underwent complete metamorphosis in response to excess K^+ (Fig. 4). The larvae were responsive over a broad range of excess K^+ concentrations. The lowest concentration of excess K^+ of 5 m*M,* which was ineffective with the other species, produced an average of 75% metamorphosis by 24 h exposure. Increasing concentrations ≥20 m*M* excess K^+ produced progressive evidence of toxicity; after 5 days at these concentrations, post-metamorphic development of the caudal tail appeared stunted.

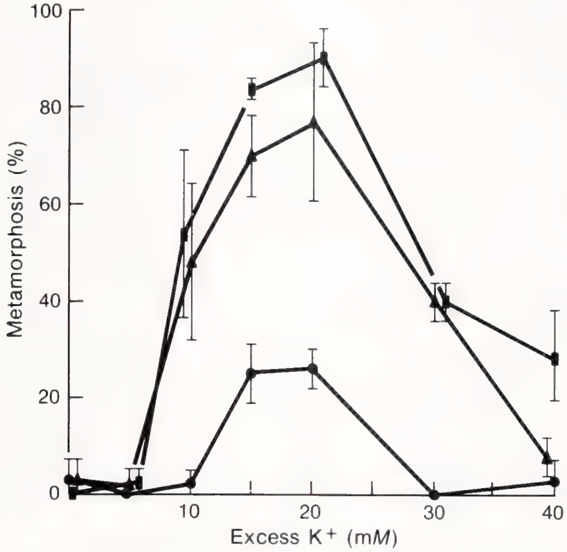

FIGURE 1. Induction of metamorphosis of larval *Phestilla sibogae* by increased external potassium. Potassium was added in excess to MBL seawater as KCl. The percentage of larvae metamorphosed was scored at 24 h (●), 48 h (▲), and 72 h (■) of continuous exposure. Data are averages of duplicates, with standard deviations indicated by vertical bars.

The effects of excess K^+ in combination with those of substratum-derived inducers or their analogs were additive. Since the response of larvae approached a maximum limit of 100% with increasing durations of exposure and higher concentrations of an

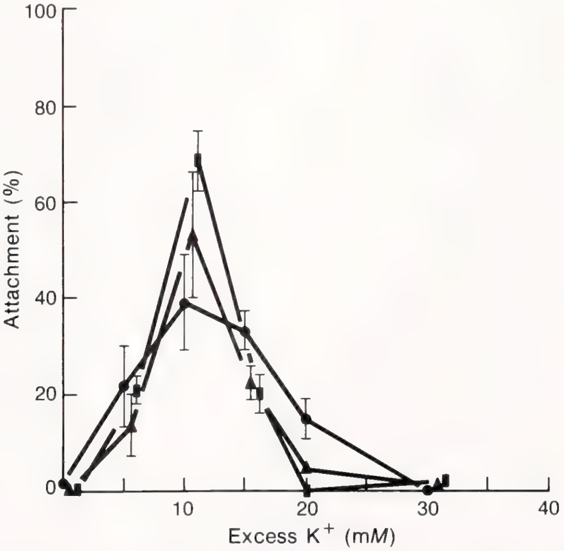

FIGURE 2. Induction of attachment of larval *Haliotis rufescens* by increased external potassium. The percentage of larvae attached was scored at 22 h (●), 48 h (▲), and 71 h (■) of continuous exposure, with other details as in the legend for Figure 1.

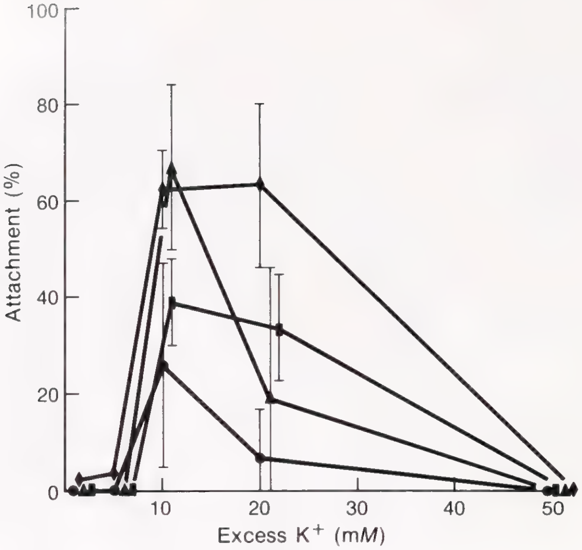

FIGURE 3. Induction of attachment of larval *Astraea undosa* by increased external potassium. The percentage of larvae attached was scored at 5 h (●), 22 h (▲), 49 h (■), and 92 h (♦) of continuous exposure, with other details as in the legend for Figure 1.

inducer, this additivity was most apparent at shorter exposure times and at lower concentrations. At 24 h, an excess of 10 m*M* K^+ nearly doubled the response of *P. sibogae* larvae to coral extract (Fig. 5). At longer durations of exposure (48 and 72 h),

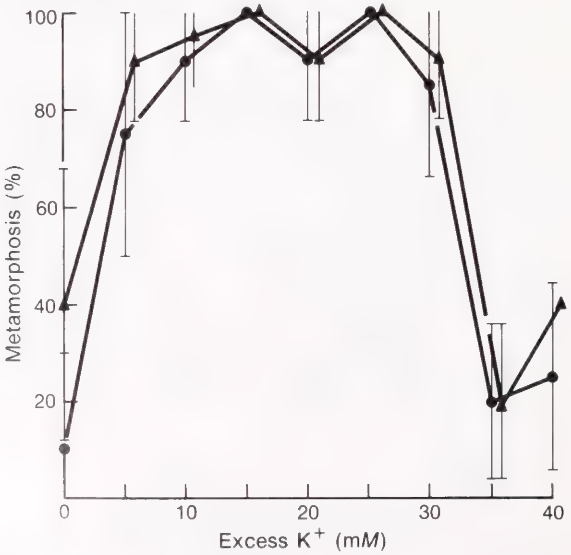

FIGURE 4. Induction of metamorphosis of larval *Phragmatopoma californica* by increased external potassium. The percentage of larvae metamorphosed was scored at 24 h (●), and 48 h (▲) of continuous exposure. Data are averages of four replicates, with standard deviations indicated by vertical bars. In parallel incubations, tube material produced by conspecific adults induced 60 (±28) % and 70 (±42) % of the larvae to metamorphose by 24 h and 48 h, respectively.

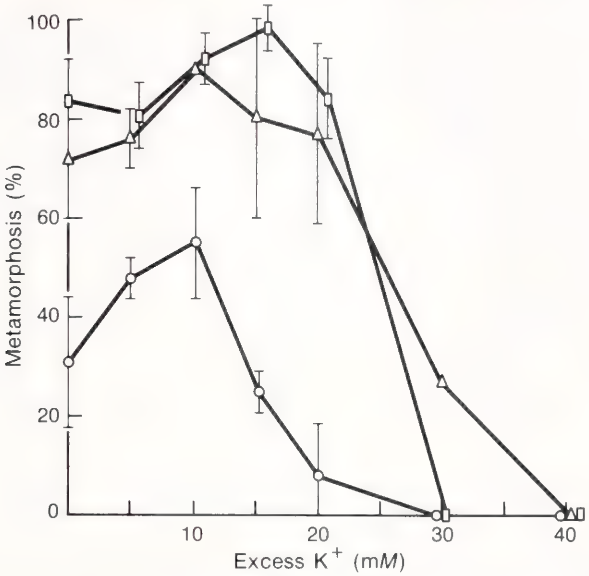

FIGURE 5. Induction of metamorphosis of larval *Phestilla sibogae* by excess potassium, when present in addition to inductive coral extract. The percentage of larvae metamorphosed was scored at 24 h (○), 48 h (△), and 72 h (□) of continuous exposure. The coral extract was prepared as described in the text, with other details as in the legend of Figure 1. For values obtained in the absence of coral extract, see Figure 1.

the percentage of larvae induced by coral extract to metamorphose was only slightly increased by excess K$^+$ (Fig. 5). The additive effect of K$^+$ and GABA as inducers of metamorphosis for *H. rufescens* was described previously (Baloun and Morse, 1984), and is presented here in more detail (Fig. 6). The presence of 5 and 10 m*M* excess K$^+$ enhanced the metamorphic response of the larvae at 22 and 48 h. The response was not greater than the sum of the responses to each inducer separately (compare Fig. 2), indicating that the additive effect of K$^+$ with GABA is not synergistic. No tests of additive effects with K$^+$ were performed with larvae of *A. undosa* or *P. californica*.

The inductive action of KCl, when added in excess to defined seawater medium, is specifically a function of the K$^+$ ion concentration, and is not dependent on an increase in osmotic pressure or paired anion concentration. Thus, comparable increases in the concentrations of other seawater cations have no inductive effect with larvae of *Haliotis rufescens* (Baloun and Morse, 1984). Competent larvae of *P. sibogae* in MBL seawater with excess NaCl (at 15, 30, or 60 m*M*), or with $CaCl_2$ or $MgCl_2$ (at 7.5, 15, or 30 m*M*), showed normal premetamorphic swimming behavior, and 0 (±0 S.D.) % of the larvae metamorphosed by 70 h exposure. Similarly, 0 (±0 S.D.) % of the larvae of *P. californica* metamorphosed in excess Na$^+$ or Mg^{2+} media (at the same concentrations as above) through 70 h; however, excess Ca^{2+} induced up to 50 (±14 S.D.) % metamorphosis by 48 h of exposure (data not shown).

Tetraethylammonium chloride (TEA), a quarternary cation known to block some K$^+$ channel currents (Armstrong, 1974; Hermann and Gorman, 1981) has been demonstrated to inhibit the metamorphic response of *H. rufescens* larvae to 12 m*M* excess K$^+$, at a concentration of 5×10^{-5} *M* TEA (Baloun and Morse, 1984). In contrast, the inductive action of excess K$^+$ with *P. sibogae* and *P. californica* larvae was insensitive to the presence of TEA, over a range of concentrations (Table IA, B). TEA did not inhibit the response of *H. rufescens* larvae to GABA (Baloun and Morse, 1984)

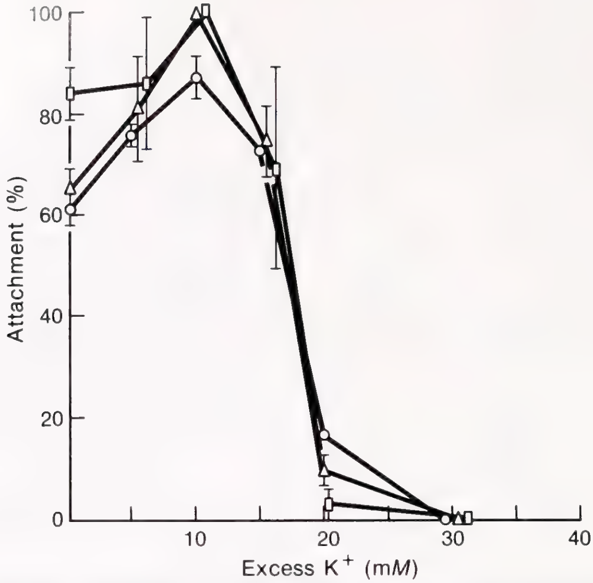

FIGURE 6. Induction of attachment of larval *Haliotis rufescens* by excess potassium, when present in addition to the chemical inducer GABA. The concentration of GABA was 4×10^{-7} *M*. The percentage of larvae attached was scored at 22 h (○), 48 h (△), and 71 h (□) of continuous exposure, with other details as in the legend for Figure 1. For values obtained in the absence of GABA, see Figure 2.

or the response of *P. sibogae* larvae to coral extract (Table IA). The effect of TEA on the response of *A. undosa* larvae has not yet been tested.

DISCUSSION

The gastropods *Phestilla sibogae, Haliotis rufescens, Astraea undosa,* and the polychaete *Phragmatopoma californica* differ in their native habitats. This difference is reflected in the dissimilar nature of the metamorphic signals recognized by the larvae. Larvae of *Phestilla,* a small subtropical carnivore, settle and metamorphose on the prey species *Porites compressa* in Hawaiian coral reefs (Hadfield, 1977). Larvae of the macroalgal herbivores *Haliotis* and *Astraea* metamorphose on substrates covered by various crustose red algae, on which the juveniles feed in the intertidal and subtidal zones of the southern California coast (Morse *et al.,* 1979; 1980a; and unpub. obs.). Larvae of the filter-feeding tubeworm *Phragmatopoma* metamorphose gregariously in response to the anterior tube material of conspecific adults (Jensen and Morse, 1984).

Certain neuroactive compounds applied externally have been demonstrated to induce larval metamorphosis in several species. These inducers are specific, and have been suggested to act either as chemical analogs of the natural substratum-derived inducers, or as precursors or active components within a signaling pathway. Choline (at 7.2×10[illegible] *M*, as choline chloride) induces metamorphosis of 60–85% of *P. sibogae* larvae (Hadfield, 1978; Hadfield *et al.,* in prep.), but has no inductive effect (at 10^{-3} *M*) on *H. rufescens* larvae (Morse *et al.,* 1979). GABA (at 10^{-6} to 10^{-3} *M*) induces virtually 100% of *H. rufescens* larvae to settle and attach, and (at 10^{-6} *M*) to complete metamorphosis (Morse *et al.,* 1979); GABA also induces settlement and metamorphosis of *Astraea undosa* larvae (Markell and Morse, in prep.) but has no inductive effect (at

TABLE I

Effect of the potassium channel blocker (TEA) on responses of larvae to excess potassium or the substrate-derived inducer

Species	Inducer[1]	Conc.[2]	TEA[3] (M)	Metamorphosis[4] (% ± S.D.)
A. *Phestilla sibogae*	None	—	0	15 ± 3
			5×10^{-6}	3 ± 3
			5×10^{-5}	1 ± 1
			5×10^{-4}	0 ± 0
	Excess K^+	20	0	71 ± 5
			5×10^{-6}	65 ± 25
			5×10^{-5}	70 ± 16
			5×10^{-4}	65 ± 17
	Coral extract	1:15	0	100 ± 0
			5×10^{-6}	94 ± 6
			5×10^{-5}	96 ± 3
			5×10^{-4}	93 ± 8
B. *Phragmatopoma californica*	None	—	0	0 ± 0
			5×10^{-6}	0 ± 0
			5×10^{-5}	0 ± 0
			5×10^{-4}	0 ± 0
	Excess K^+	15	0	80 ± 0
			5×10^{-6}	80 ± 0
			5×10^{-5}	80 ± 28
			5×10^{-4}	90 ± 14
	Conspecific tube material	—	0	80 ± 0

[1] All tests in MBL seawater. Excess K^+ added as KCl; extract prepared from coral as described in text.

[2] Concentration of excess K^+ in mM; coral extract diluted 1 part to 15 parts MBL seawater.

[3] TEA (tetraethylammonium chloride).

[4] Larval metamorphosis at 48 h, averaged from triplicate samples for *P. sibogae,* and duplicate samples for *P. californica.*

9.7×10^{-3} or 9.7×10^{-2} M) on *P. sibogae* larvae (Hadfield, 1984). Certain catecholamines show different activities as inducers of metamorphosis in larval *P. californica* (Jensen and Morse, unpub. obs.), and cause partial metamorphosis of larval *P. sibogae* (Hadfield, 1984), but have no inductive effect on *H. rufescens* (at 10^{-3} M; Morse *et al.,* 1979) or on *A. undosa* (Markell and Morse, in prep.). Catecholamines also have been shown to induce metamorphosis of the sand dollar *Dendraster excentricus* (Burke, 1983a), and the oyster *Crassostrea gigas* (Coon *et al.,* 1984).

In sum, the external chemical stimuli capable of inducing larvae of *Phestilla, Haliotis, Phragmatopoma,* and *Astraea* to metamorphose are restricted in activity by species-specificity, with the notable exception of potassium. As reported here, larvae of four species from two invertebrate phyla share a sensitivity to K^+ as an inducer of metamorphosis. Excess K^+ also has been found to induce metamorphosis of the urchin, *Lytechinus variegatus* (Cameron and Tosteson, in prep.). The inductive effect of external cations on larval metamorphosis was demonstrated previously by Spindler and Müller (1972) and Müller and Buchal (1973), who showed that planula larvae of *Hydractinia echinata* (Cnidaria) metamorphosed in response to increased K^+, Li^+, Rb$^+$ and [illegible]. With the protocol used by Müller and colleagues (involving short exposures of larvae to high doses), K$^+$ proved to be the least effective inducer among the cations tested. Nonetheless, their result appears to support the generality of the effect of potassium by extending the examples of responsive larvae to include species from four invertebrate phyla.

Since membrane potential in excitable cells is influenced by changes in the K$^+$ electrochemical gradient (Hodgkin and Horowicz, 1959; Hagiwara *et al.,* 1961; Monticelli, 1979; Schlue and Deitmer, 1984), we have suggested that increased external K$^+$ may activate metamorphosis by depolarizing externally accessible cells in an inductive pathway (Baloun and Morse, 1984). The magnitude of the depolarization resulting from the doubling of external K$^+$ concentration (generally found optimal in our experiments), as estimated from the Nernst equation, is on the order of $^+$17 mV. This putative depolarization appears to bypass the species-specific receptor-stimulus interaction, as verified both by pharmacological analyses that resolve the two effects in *Haliotis rufescens* larvae (Baloun and Morse, 1984), and by recent experiments directly characterizing the down-regulation of the chemosensory receptors controlling metamorphosis in this species (Trapido-Rosenthal and Morse, submitted).

Data reviewed previously indicate that both the chemosensory receptors and the potassium-depolarizable cell membranes that control settlement and metamorphosis of *H. rufescens* are located on the epithelial surface of the larvae (Baloun and Morse, 1984; Morse, 1986). These larvae also are found to be unaffected by tetrodotoxin, suggesting that the epithelia are not leaky (Baloun and Morse, 1984). In the experiments reported here, the technique involving total immersion of larvae in modified seawater media prevents a localized application of the potassium stimulus. It is possible, then, that excess K$^+$ might influence a variety of cells exposed on the surface of the larva, and induces metamorphic changes independently in different target tissues. However, the complex metamorphic changes in behavior, loss of larval specializations, and new growth of adult structures follow the same critical sequence whether induced by excess K$^+$ or by the natural substratum. Thus, we believe it more likely that depolarization with K$^+$ activates the normal morphogenetic pathway, which in turn coordinates the genetically programmed sequence of behavioral and developmental changes resulting in metamorphosis. The ability of excess K$^+$ to effect a rapid change in behavior from the swimming premetamorphic phase to the crawling juvenile phase, without affecting the behavioral responses of larvae to light and mechanical stimuli, also argues for a restricted effect of K$^+$ on the larvae.

The potassium channel blocker, TEA, inhibits the induction of metamorphosis by excess K$^+$ in larval *H. rufescens* (Baloun and Morse, 1984). The insensitivity of *P. sibogae* and *P. californica* larvae to TEA (Table IA, B) indicates that K$^+$ may act through channels different from those suggested to be involved in the potassium-induced metamorphosis of *H. rufescens* larvae (Baloun and Morse, 1984). Several classes of physically distinct channels that conduct K$^+$ currents in molluscan neurons have been identified in studies of their current kinetics, gating dependence, pharmacological sensitivity, and selectivity (Thompson, 1977; Westerfield and Lux, 1982; review by Edwards, 1982). Pharmacological analyses have shown that channels conducting potassium currents include those which are TEA-insensitive (Kostyuk *et al.,* 1980; Edgington and Stuart, 1981), blocked by internally applied TEA (Armstrong and Binstock, 1965), or sensitive to both internal and external TEA (Hermann and Gorman, 1979; 1981). Thus it appears possible that potassium channels mediate the induction of metamorphosis by K$^+$ in *P. sibogae* and *P. californica,* but that K$^+$ conductance through these channels is insensitive to external TEA.

The effectiveness of potassium does not imply that K$^+$ currents necessarily are involved in transducing natural signals for metamorphosis. In fact, the activation of larval receptor cells by depolarization could be accomplished by cation influx or anion efflux involving any of the physiologically relevant ions. For example, the response of *H. rufescens* larvae to GABA may depend in part on a depolarizing efflux of chloride (Baloun and Morse, 1984). In addition, the observation made here that *P. californica* larvae metamorphose in excess Ca^{2+} medium may suggest a role for calcium in signal transduction in these larvae. Calcium fluxes also have been implicated in the modulation or transduction of the morphogenetic signal in *H. rufescens* larvae (Morse *et al.,* 1980a; Baloun and Morse, 1984). Thus, while the common requirement of signal transduction following receptor activation may be depolarization, the mechanisms driving and responding to this event may vary among diverse species.

The results presented here demonstrate the effectiveness of potassium as a morphogenetic inducer in larvae of four marine invertebrate species. Subsequent electrophysiological and biochemical studies employing this effect should prove useful to further characterize the molecular, cellular, and neuronal mechanisms involved in substratum recognition, and the control of larval settlement and metamorphosis.

ACKNOWLEDGMENTS

We wish to thank N. Hooker and D. Lauro for their invaluable assistance with larval culture, and H. Trapido-Rosenthal for helpful discussions. This research was supported in part by the U. S. Navy Office of Naval Research (Contract #N00014-80-C-0310 to D.E.M.), the National Science Foundation (Grants #DCB-8415411 to D.E.M., and #PCM-8215552 to M.G.H.), and the International Women's Fishing Association (Graduate Fellowship to A.J.Y.). This assistance is most gratefully acknowledged.

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EVIDENCE FOR HETEROTROPHY BY ZOOXANTHELLAE IN SYMBIOSIS WITH *AIPTASIA PULCHELLA*

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ABSTRACT

Sea anemones (*Aiptasia pulchella*) fed ^{35}S methionine labeled food translocated labeled material to symbiotic zooxanthellae. This uptake of host-derived organic materials by the symbiotic algae is interpreted as evidence for heterotrophy. It is calculated that 2.8–6.4% of the zooxanthellae growth requirement for protein was satisfied by heterotrophy under a 12 hour light:12 hour dark photoperiod. The cost of algal heterotrophy to the animal host was demonstrated by exposing aposymbiotic anemones to cultured zooxanthellae. Aposymbiotic animals rapidly re-established a stable symbiosis, but the symbiotic animals had a protein biomass significantly lower than control aposymbionts, suggesting that the reinfection of the symbiotic animals by zooxanthellae caused a decrease in animal biomass. Zooxanthellae spontaneously appeared and grew in the control aposymbionts. These repopulating algal cells were apparently derived from a residual heterotrophic population of zooxanthellae maintained by anemones after four years of darkness. Aposymbiotic anemones starved in darkness suffered a lower mortality rate than symbiotic animals under the same conditions, suggesting that heterotrophic symbiotic zooxanthellae can impose a fatal metabolic burden on these anemones.

INTRODUCTION

The metabolic relationship between intracellular dinoflagellate symbionts (= zooxanthellae; *Symbiodinium microadriaticum*) and their cnidarian hosts has been investigated extensively from the perspective of benefit of the algae to the animal. Zooxanthellae enhance the survival of symbiotic animals relative to that of aposymbiotic animals when both are starved in light (Fitt and Pardy, 1981). Zooxanthellae within host cells are photosynthetic, and tracer experiments have demonstrated that ^{14}C labeled algal photosynthate is translocated to the animal cells, where it is respired or incorporated into host proteins and lipids (Trench, 1981; Battey and Patton, 1984). Translocated carbon can potentially supply 20–150% of the carbon required for animal respiration (Muscatine *et al.,* 1983; McCloskey and Muscatine, 1984; Steen and Muscatine, 1984).

Little attention has been given to the possible benefit of symbiosis to the alga or the cost of symbiosis to the animal partner. Recently Douglas and Smith (1983) found that symbiotic hydra grew more slowly in darkness than aposymbiotic animals when both groups were given equal food rations, suggesting that zoochlorellae constitute a metabolic cost to the host in darkness. Several studies suggest that symbiotic algae are capable of uptake of organic material derived from the host. For example, Cook (1971) fed ^{35}S methionine labeled food to *Aiptasia* sp. and demonstrated that radioactively

Received 30 October 1985; accepted 14 January 1986.

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labeled substrates moved from host to algae, however the quantitative significance of this "reverse translocation" into the symbiotic algae was not assessed. Thorington and Margulis (1981) showed that when *Hydra viridis* was fed *Artemia* nauplii labeled with tritiated precursors of protein and nucleic acids, labeled tritium appeared in algal and animal fractions in a manner suggesting that algae and host might be competing for limited nutrients. However tritium is probably an unsuitable radionuclide for this type of study because proton exchange reactions are ubiquitous in living systems (Zubay, 1983). Cook (1983) has noted that ^{14}C would also be an inappropriate radiolabel for a reverse translocation study because of the possible metabolism of ^{14}C labeled organic substrates by the animal and fixation of the resulting $^{14}CO_2$ by algal photosynthesis.

In vitro experiments suggest that cultured zooxanthellae algae are capable of uptake of organic compounds potentially present in the host (Steen, 1985). Zooxanthellae freshly isolated from the anemone *Cassiopea xamachana* have an active transport mechanism for alanine uptake which is inhibited by a protein fraction present in host homogenate (Carroll and Blanquet, 1984). Cultured zooxanthellae from *Tridacna maxima* have active transport mechanisms for cysteine, methionine, and taurine (Deane and O'Brien, 1981).

These previous studies suggest that growth of symbiotic zooxanthellae is potentially augmented through uptake of host-derived organic compounds. This paper addresses the questions of whether algal heterotrophy occurs in symbiosis and whether the animal host incurs a metabolic cost from the maintenance of a population of symbiotic zooxanthellae.

I report evidence that symbiotic algae in a sub-tropical sea anemone can grow through heterotrophic uptake of host-derived organic materials even in light. Anemones with a rapidly growing population of zooxanthellae have lower protein biomass than normal aposymbiotic anemones, suggesting that the host incurs a metabolic cost from algal growth. Consistent with this, I report that symbiotic anemones starved in darkness have a higher rate of mortality than aposymbiotic anemones starved under the same conditions.

MATERIALS AND METHODS

Animal maintenance

One clone of sea anemones (*Aiptasia pulchella*) derived from a single animal originally collected in Kaneohe Bay, Hawaii, by G. Muller-Parker, was maintained in a 10 gallon Plexiglas tank of aerated seawater at room temperature (20–25°C) exposed to indirect sunlight (50–100 $\mu Ein \cdot m^{-2} \cdot s^{-1}$). Animals were fed *Artemia* nauplii to repletion twice weekly and the tank was cleaned monthly. Anemones used in experiments were randomly selected, then segregated into several one liter finger bowls in 800 ml of seawater. Animals were starved for 3–4 days before use in experiments. During experiments, anemones were maintained in a controlled temperature incubator at 25°C ± 1° under 70 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ irradiance (12 hour light:12 hour dark photoperiod) except as noted. Bowls of animals maintained under high irradiance were placed in a 25°C Lauda Brinkmann circulating water bath under a 500W Sylvania tungsten halogen "very wide flood" lamp that provided 300 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ of light incident on the animals.

Aposymbiotic anemones were obtained by "cold stripping": normal symbiotic anemones were induced to extrude 99% of their symbiotic algae within one week by exposure to 4°C for 4 hours and subsequent maintenance in darkness. This technique produces no animal mortality (Steen and Muscatine, in prep.). Some experiments used aposymbiotic anemones produced by prolonged (>4 years) culture in the dark.

Labeling animal food with ^{35}S methionine

A stock of 0.1 ml of 10 mCi · ml^{-1} ^{35}S-methionine in ethanol (New England Nuclear) was diluted to 1 ml with rat Ringers solution and the entire quantity was injected into the tail vein of an anesthetized nine month old female Sprague-Dawley rat. After three days the rat was sacrificed, and the internal organs (heart, liver, lungs, kidneys, and intestines) were removed and assayed for tissue specific activity (radioactive disintegrations · $minute^{-1}$ · mg $protein^{-1}$). Protein was measured by the method of Lowry *et al.* (1951). The intestines were the most heavily labeled tissue and were therefore used in all feeding experiments. Intestines were stored at −20°C until used.

The percent of total rat intestine ^{35}S-methionine in protein (rather than in intracellular free amino acid pools) was determined from sequential hot water extractions of rat intestine. About 65% of total radioactivity was hot water soluble, with 70% of the extractable counts removed during the first extraction. More extensive extraction led to tissue disintegration and a preparation unsuitable for feeding anemones. Therefore intestine was extracted only once in hot water, so that, of the total ^{35}S intestine fed to anemones, approximately 65% was particulate, with the remainder being unextracted soluble ^{35}S.

Translocation of ^{35}S-labeled compounds from host to algae

Four random samples of symbiotic anemones were segregated from a single population, and one of these groups was rendered aposymbiotic by "cold stripping." Each anemone was then individually fed a single 5–6 mm^2 piece of flattened rat intestine previously extracted once with hot water. Animals were allowed two hours in light for ingestion. Uningested food was then removed and the water in the bowls was replaced with fresh sea water before animals were placed in the incubator. Seawater was replaced initially after 4–6 hours to remove freshly egested food, then water was replaced daily in the animal bowls. After three days animals were individually homogenized in 10 ml of seawater in a glass tissue grinder with a motor-driven Teflon pestle. Zooxanthellae were separated by centrifugation for three minutes at 500 × *g* in a Damon/IEC HN-S clinical centrifuge. One ml aliquots of animal homogenate were placed in scintillation vials, dried at low heat on a hot plate, and solubilized in toluene-Triton X scintillation fluor. The algal pellets were washed three times by resuspension and centrifugation in seawater, then placed in a scintillation vial, and dried. Ten ml of fluor was added to the dried remains and the vials were stored overnight in darkness before scintillation counting. Radioactivity was measured in a Beckman LS 100C scintillation counter and all counts were background and quench corrected. Radioactive disintegrations per minute (dpm) for each sample were calculated with ^{35}S half-life corrections. The same procedure was also followed using aposymbiotic anemones.

Reinfection of aposymbiotic animals with cultured zooxanthellae

Anemones which had been rendered aposymbiotic by prolonged (>4 years) maintenance in darkness, were randomly assigned to two groups of 12 animals each. One group was exposed to a 1 ml innoculum of 10^6 cultured zooxanthellae (CZ) cells (Steen, in prep.), washed and resuspended in Millipore-filtered (0.45 μ pore size) seawater, then added to the bowl in which animals were maintained. The other group was incubated without exposure to CZ. Both groups were maintained in Millipore-filtered seawater which was changed daily. A third group of similar size light-reared symbiotic anemones was segregated and all three groups were maintained in an incubator at 36 μEin · m^{-2} · s^{-1} for 17 days. Animals were then individually homogenized and the zooxanthellae separated as before. Anemone biomass was measured as animal protein by the Lowry technique (1951) and number of zooxanthellae was normalized to animal protein. Algal mitotic index was measured (Wilkerson *et al.*, 1983) at 430× on a hemocytometer slide using an Olympus BH-2 microscope and cell specific growth rate was calculated after McDuff and Chisholm (1982).

Survival of starved animals

Twenty-five individuals each of symbiotic and aposymbiotic anemones were randomly selected and placed in 1 liter finger bowls. Both groups of anemones were starved in darkness at 25°C. Seawater was changed twice weekly and animals were censused at the same time. Dead animals were seldom observed, because animals shrank during starvation and animals tended to dissolve upon death. The experiment was terminated at week 15 and the "symbiotic" anemones were individually homogenized, the zooxanthellae collected by centrifugation, and the number of zooxanthellae per anemone calculated from hemocytometer counts of aliquots of the resuspended zooxanthellae.

RESULTS

Translocation of ^{35}S label from host to algae

Direct evidence for "reverse translocation" was obtained by feeding symbiotic animals with radioactive food and measuring the subsequent appearance of label in the zooxanthellae. The average total dpm of food given to each anemone was 9014.4 ± 1957.7 dpm. The specific activity of the food was 486.7 dpm per 1 μg protein (see Discussion), so the average meal was 18.5 μg protein.

The average assimilation of ^{35}S-methionine label into all groups of symbiotic animals after three days averaged 21.6% (Table I), calculated by dividing dpm assimilated by the average activity of ingested food particles. Since anemones had been randomly

TABLE I

Assimilation of ^{35}S label by Aiptasia pulchella *and symbiotic zooxanthellae after a three day incubation*

	Total ^{35}S dpm assimilated per anemone	Average assimilation efficiency (%)	Percent total assimilated dpm in algal pellet
Constant light	1771.3 (±968.6)	19.67	10.17 (±0.16)
Constant darkness	2264.4 (±1079.1)	25.07	10.82 (±0.22)
Light/dark photoperiod	1810.6 (±865.6)	20.09	12.26 (±0.37)
Aposymbiotic anemones (darkness)	3859.8 (±1568.1)	42.82	6.53 (±0.16)

Animals were fed pieces of ^{35}S-methionine labeled rat intestinal tissue. Incubator irradiance was 70 μEin · m^{-2} · s^{-1} in the light experiments. All animals were symbiotic except the aposymbiotic dark control. Activity is shown as disintegrations · min^{-1} (dpm) ± standard deviation. Results shown are pooled from two separate experiments with a total sample size of n = 27 animals in every case except the experiment with aposymbiotic anemones where n = 15. Pellet-associated radioactivity is ^{35}S dpm in zooxanthellae and/or in animal tissue contaminants of the algal pellet.

selected from a single population, it is assumed that the average animal biomass of each group of anemones was equivalent. Therefore the extent of ingestion and assimilation of ^{35}S methionine label differs between groups because of the treatments, not because of anemone size differences. Dark-maintained animals assimilated significantly more radioactive label than did animals in constant light or on a 12 light:12 dark photoperiod (*t*-test; $P < 0.05$). Aposymbiotic animals assimilated significantly more label than did any of the symbiotic animal groups (*t*-test; $P < 0.01$). It was observed that aposymbiotic animals held food in the gut for as long as 24 hours, while symbiotic anemones tended to egest food within 6 hours.

In constant light and constant darkness appearance of dpm in the algal pellet was 10.2% and 10.8%, respectively, of total dpm assimilated, while the appearance of dpm in the zooxanthellae of anemones on a light/dark photoperiod was 12.3%. Since pellet-associated ^{35}S could arise from ^{35}S labeled animal tissue contaminants as well as algal assimilation of ^{35}S methionine, the radioactivity in the aposymbiotic animal pellet was used as a control for pellet contaminants of animal origin. The percentage of pellet-associated radioactivity was significantly lower in aposymbiotic than in all groups of symbiotic animals (arcsine transformed data used for *t*-test; $P < 0.0005$) (Zar, 1974).

The largest proportion of total activity in the algal pellet was found in animals on a light/dark photoperiod (arcsine transformed data used for *t*-test; $P < 0.025$). After correction for animal tissue contaminants in the algal pellet, the proportion of zooxanthellae-associated ^{35}S-methionine radioactivity was 3.6%, 4.3%, and 5.7% in anemones maintained in constant light, constant darkness, or on a photoperiod, respectively. Movement of ^{35}S label into the algal pellet is interpreted as heterotrophic uptake by the zooxanthellae. However the measured amount of ^{35}S label in zooxanthellae isolated from dark-maintained anemones may underestimate the extent of algal heterotrophy in this case, because *A. pulchella* rapidly extrude pellets of symbiotic zooxanthellae in darkness (Steen and Muscatine, in prep.). Results presented here do not exclude the possibility that such pellets were radioactively labeled, leading to an underestimate of the total extent of ^{35}S incorporation into zooxanthellae symbiotic with dark-maintained hosts.

Reinfection of aposymbiotic animals

To assess cost to the host of a growing population of symbiotic algae, aposymbiotic animals were reinfected with CZ. *A. pulchella* were fed twice weekly in darkness for four years at which time microscopic examination revealed no pigmented cells with recognizable zooxanthellae morphology. Seventeen days after innoculation with CZ these animals re-established the relationship so that no significant differences were observed between symbiotic (control) and reinfected animals in numbers of zooxanthellae per anemone or zooxanthellae density (Table II). However both standing stock and density of zooxanthellae were significantly higher in reinfected animals than in light-maintained aposymbiotic control animals which did not receive an inoculum of CZ (Wilcoxon two-sample test; $P < 0.001$). Therefore CZ were able to repopulate aposymbiotic hosts and to reach densities comparable to zooxanthellae in normal light-reared animals.

Survival of starved animals

Aposymbiotic and symbiotic animals were starved in darkness to ascertain whether and to what extent the presence of potentially heterotrophic symbiotic algae affected the survival of the host. Figure 1 shows that, during the 15 week starvation period,

TABLE II

Reinfection of aposymbiotic Aiptasia pulchella *with cultured zooxanthellae*

	Anemone biomass 17 days after exposure to CZ (mg protein · anemone^{-1})	Zooxanthellae per anemone ($\times 10^5$)	Zooxanthellae cell density ($10^5 \cdot$ mg animal prot^{-1})	Algal mitotic index (% dividing cells)	Cell specific growth rate (day^{-1})
Uninoculated apo control anemones n = 12	1.039 (±0.22)*	1.11 (±1.78)*	0.94 (±1.35)*	—	—
Inoculated apo anemones n = 12	0.623 (±0.26)	5.82 (±3.55) NS	10.41 (±6.87) NS	1.05 (±0.22)	0.0514
Light-reared sym anemones n = 12	0.885 (±0.37)	7.72 (±6.07)	7.87 (±3.61)	0.85 (±0.15)	0.0416

* Includes four anemones which spontaneously reinfected with zooxanthellae to levels comparable to sym anemones.

Aposymbiotic (apo) animals were maintained for 17 days in Millipore-filtered (0.45 μ) seawater changed daily at 25° ± 1°C under an irradiance of 56 μEin · m^{-2} · s^{-1}. Aposymbiotic animals were obtained from G. Muller-Parker and the control group was comprised of similar size light-reared symbiotic (sym) anemones. Animals were individually homogenized at the end of 17 days and zooxanthellae were separated by centrifugation. Anemone biomass was measured as animal Lowry protein and zooxanthellae per anemone is expressed as the number of cells per mg animal protein. Algal mitotic index was measured from 10 samples of 100 zooxanthellae each. Values shown are mean ± standard deviation. NS indicates no significant difference between values.

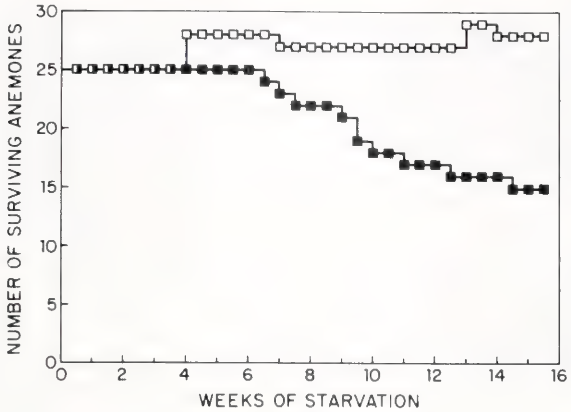

FIGURE 1. Survival of dark-maintained starved symbiotic (■) and aposymbiotic (□) *A. pulchella.* The aposymbiotic animal population underwent a slight increase because of the production of new pedal lacerates. Symbiotic animals progressively declined in number after week 6.

aposymbiotic animals starved in darkness produced pedal lacerates thereby increasing the total number of individuals present. The death of a single aposymbiotic anemone at week 7 was a newly produced pedal lacerate. No further deaths occurred in the aposymbiotic population until week 14, when another new pedal lacerate died. Although no quantitative data is available, the aposymbiotic animals appeared to be larger at the end of the experiment than the symbiotic animals.

The survival of symbiotic animals starved in darkness was significantly lower than that of aposymbionts (log-rank method adapted from Azen *et al.*, 1977; $\chi^2 = 12.27$; $P < 0.001$). No pedal lacerates were produced and the first death (disappearance) of a symbiotic animal occurred at week 6. After week 6 symbiotic animals died at the rate of more than one a week for 6 weeks. During this period of starvation symbiotic animals progressively lost symbiotic algae. The overall percentage of survival at week 15 was 60% for symbiotic anemones and 112% for aposymbionts. The experiment was terminated at week 15 because most of the surviving symbiotic animals were depleted of zooxanthellae. The average number of zooxanthellae per anemone in the symbiotic animals at the end of the experiment was 0.17×10^5, 98% fewer zooxanthellae than a similar light-reared control (Table II).

DISCUSSION

Reverse translocation

The ^{35}S experiments suggest that uptake of host-derived organic materials by zooxanthellae occurs in laboratory-maintained *A. pulchella.* Cook (1971) first used ^{35}S-methionine labeled protein to show movement of radiolabel from *Aiptasia* sp. into symbiotic zooxanthellae. However the magnitude of reverse translocation reported (Cook, 1971) was higher and more variable than reported here. Five days after feeding *Aiptasia* sp. with ^{35}S labeled food, approximately 31% (±10% standard deviation) of the total recovered radioactivity was associated with the algal pellet (Cook, 1971). However no indication was given of the extent of animal tissue contamination in the algal pellet. In the present study, such contamination was observed to be greater than 50% of the pellet-associated radioactivity.

The average assimilation efficiency of ^{35}S methionine-labeled protein by *A. pulchella* was 21.1% (Table I). This figure is low compared to the 61% digestive assimilation efficiency of *A. pulchella* calculated from the ratio of caloric loss in feces to caloric intake in food (Hunter, 1984). However this ratio does not include caloric loss in dissolved or microparticulate organic matter, so it is possible that assimilation efficiency was overestimated (Hunter, 1984). Preliminary results (Steen, unpub.) suggest that the average efficiency of assimilation of ^{35}S methionine dissolved in seawater into animal tissue is considerably less than 1%. This suggests that assimilation of ^{35}S methionine into anemone tissue was mainly assimilation of food protein rather than assimilation of pools of free methionine in the food particles.

The contribution of heterotrophy to algal growth (CHAG) was calculated using the data for reverse translocation of ^{35}S-methionine in animals on a 12L:12D photoperiod (Table I). Standing stock of algae in *A. pulchella* was 0.77×10^6 zooxanthellae per anemone (Table II; light reared control) and the cell specific growth rate for zooxanthellae within the host was calculated as $\mu = 0.0416 \cdot \text{day}^{-1}$ (Table II; light reared control) using $t_d = 4.88$ hours (Steen, 1985). The number of new zooxanthellae cells produced during three days of the experiment was;

$$\begin{aligned}\text{New cells} &= 3\mu \times \text{standing stock of zooxanthellae}\\ &= 9.61 \times 10^4 \text{ zooxanthellae}\end{aligned}$$

Lowry protein assays of freshly isolated washed zooxanthellae showed there was 78.5 μg protein per 10^6 zooxanthellae. The product of the number of new cells and the protein per cell equals the quantity of new zooxanthellae protein synthesized during the experiment.

$$\begin{aligned}\text{New zooxanthellae protein} &= 78.5 \times 0.0961\\ &= 7.5439\ \mu\text{g protein} \qquad (1)\end{aligned}$$

The proportion of total radioactivity incorporated into zooxanthellae was calculated as 5.73%, the difference between the light:dark photoperiod algal pellet and the aposymbiont pellet (Table I). Therefore;

$$\begin{aligned}\text{Zooxanthellae-associated dpm} &= \text{total dpm assimilated} \times 0.0573\\ &= 103.7 \text{ dpm}\end{aligned}$$

The specific activity of ^{35}S-methionine labeled rat intestine was 486.7 $\text{dpm} \cdot \mu\text{g protein}^{-1}$ (regression not shown; $r = 0.92$). Assuming that the specific activity of zooxanthellae protein was identical to the specific activity of food protein and further assuming that all ^{35}S in plant cells is incorporated into protein (*cf.*, Giovanelli *et al.*, 1980), it is possible to calculate the approximate net amount of ^{35}S-labeled zooxanthellae protein produced in three days.

$$\begin{aligned}^{35}\text{S-labeled protein synthesized} &= 103.7/486.7\\ &= 0.2131\ \mu\text{g} \qquad (2)\end{aligned}$$

The contribution of heterotrophy to algal growth is calculated as (2) divided by (1) or;

$$(^{35}\text{S protein/total new protein}) \times 100 = 2.82\%$$

Therefore almost 3% of zooxanthellae growth, assayed by protein synthesis was satisfied by reverse translocation of ^{35}S-methionine, although the irradiance permitted net algal photosynthesis (Muller-Parker, 1984). This estimate is likely to be conservative because the zooxanthellae growth rate was calculated assuming $t_d = 4.88$ hours (Steen, 1985). If the value used for duration of mitosis is $t_d = 11$ hours (Wilkerson *et al.,* 1983), the estimate of CHAG becomes 6.39%. This estimate of algal heterotrophy may be further biased by the assumption that all new zooxanthellae cells produced by mitosis are retained by the host, rather than being expelled. O. Hoegh-Guldberg (pers. comm.) has shown that the rate of expulsion of symbiotic zooxanthellae may be as high as 26% of the rate of production of new zooxanthellae by mitosis. If newly produced cells labeled with ^{35}S are expelled, this would lead to an underestimate of the extent of heterotrophy.

It is possible that the zooxanthellae in this experiment became ^{35}S labeled by uptake and assimilation of inorganic rather than organic sulfur. Cultured *Tridacna* zooxanthellae are capable of active transport of sulfate as well as cysteine, methionine, and taurine (Deane and O'Brien, 1981). However methionine, in plants and animals, is chiefly utilized as a component of protein, methionyl tRNA, or S-adenosyl-L-methionine (Giovanelli *et al.,* 1980). Moreover the oxidative metabolism of sulfur-containing amino acids to sulfate is slow (Sinensky, 1977), and is therefore not relevant to the present study.

About 90% of the total sulfur in plant tissue is in the amino acids methionine and cysteine, and nearly all of these amino acids are in protein (Giovanelli *et al.,* 1980). Thus it is reasonable to assume that most of the ^{35}S activity in zooxanthellae was incorporated into protein and that the specific activity of ^{35}S labeled zooxanthellae protein was roughly equivalent to the specific activity of food protein.

The data on zooxanthellae-associated ^{35}S (Table I) suggests that heterotrophic uptake and/or growth in darkness or on a photoperiod is stimulated relative to zooxanthellae maintained in constant light. Natural populations of marine and freshwater algae synthesize protein during darkness, and night time net protein synthesis is often nearly equal to protein synthesis in light (Cuhel *et al.,* 1984). Furthermore it has been demonstrated that sulfate incorporation into protein in algae measured net protein synthesis rather than turnover (Cuhel *et al.,* 1984), suggesting that ^{35}S methionine incorporation is a reasonable measure of cell growth. Cuhel *et al.* (1984) report that sulfate uptake by *Dunaliella* is only slightly stimulated by light, and that cultures of freshwater algae may incorporate sulfate more rapidly in darkness than in low light.

Cultures of the diatom *Phaeodactylum tricornutum* maintained under conditions of environmental stress (such as low irradiance levels) conserved the ability to synthesize protein more than the ability to synthesize lipids or polysaccharides (Morris *et al.,* 1974). This increased protein synthesis was generally at the expense of the algal polysaccharide fraction (Morris *et al.,* 1974). A similar phenomenon may have been occurring with zooxanthellae in symbiosis; perhaps the allocation of heterotrophically derived carbon to protein synthesis increases proportionally as the zooxanthellae become light limited. Since uptake of methionine by zooxanthellae is an active process (Deane and O'Brien, 1981), heterotrophic uptake in constant darkness may be less than that in cells maintained on a photoperiod because the zooxanthellae can become energy-limited in darkness.

Results presented here suggest that reverse translocation or algal heterotrophy is possible in an intact symbiosis. Heterotrophy was greatest when zooxanthellae were exposed to a photoperiod similar to that which symbiotic organisms experience in the field. However the animals in this experiment experienced an irradiance much lower than natural ambient light. Muller-Parker (1984) found that the compensation irradiance of intact *A. pulchella* was 32 $\mu\text{Ein} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, but that algal photosynthesis did not saturate below 400 $\mu\text{Ein} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Experimental animals were irradiated with 70 $\mu\text{Ein} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, and thus experienced less than 20% of saturating irradiance (Muller-Parker, 1984). It is not known to what extent algal heterotrophy would be affected by ambient irradiance.

Reinfection of aposymbiotic animals

To determine if zooxanthellae within hosts can survive by heterotrophic uptake of host-derived organic material, I analyzed two independent sets of observations in the reinfection experiment. After the light incubation period one third (4 of 12) of the uninoculated aposymbiotic animals (Table II) contained recognizable zooxanthellae; these were probably derived from an undetected population of algae which had survived four years of darkness. Since animals had been fed in the dark it seems likely that zooxanthellae had survived by using materials from food ingested by the animal.

Table II also shows that the zooxanthellae which repopulated aposymbiotic animals were growing significantly more rapidly than zooxanthellae in normal light-reared anemones ($P < 0.01$) based on their mitotic index. This indicates that hosts may have become repopulated with zooxanthellae to a level comparable with normal symbiotic hosts through the rapid growth of zooxanthellae in the unexploited habitat presented by an aposymbiotic host. Reinfected animals had significantly lower protein biomass than aposymbiotic animals ($P < 0.005$) because the metabolic cost to the host may be particularly high when symbionts undergo a period of relatively rapid growth.

Survival of starved animals

The metabolic cost of zooxanthellae to a symbiotic animal is most clearly manifested in the survival curves for symbiotic and aposymbiotic animals (Fig. 1). Anemones were not weighed during this experiment, but the weight loss experienced by reinfected animals (Table II) suggests that the symbiotic animals died first because of the depletion of organic materials caused by zooxanthellae heterotrophy. These results are in contrast with data from *Anthopleura elegantissima;* normal symbiotic anemones starved in darkness lost proportionally less reduced weight than albino anemones starved in darkness (Muscatine, 1961; Fitt and Pardy, 1981).

The interaction between zooxanthellae and *A. pulchella* is dynamic, and may change in character from a commensalism or a mutualism to a parasitism depending upon environmental conditions. The dynamic nature of the relationship is demonstrated by the observation that *A. pulchella* is capable of expelling zooxanthellae in periods of prolonged darkness (Steen and Muscatine, in prep.), while zooxanthellae can grow rapidly in an unexploited host (Table II).

Ecological considerations

Caution must be used when comparing the results of studies on different symbiotic hosts, because it is likely that symbiotic zooxanthellae are composed of several strains or species (Blank and Trench, 1985). Consequently the relationship between different hosts and their symbionts may vary considerably. However if zooxanthellae are generally capable of facultative heterotrophy, how important is heterotrophy in the field? Corals or anemones growing at great depth or high latitude experience attenuated solar irradiance such that algae may be light limited. Dustan (1982) has even suggested zooxanthellae are normally light-limited in their natural habitat, as photosynthetic light saturation of isolated zooxanthellae always occurred at higher irradiance than was measured at the collection depth at local noon. The depth of penetrance of 1% of the total surface irradiance is generally accepted as the algal photosynthetic compensation depth, yet nine species of corals have been reported to maintain symbiotic zooxanthellae at great depths even though these zooxanthellae are seemingly unable to be phototrophic (Fricke and Schuhmacher, 1983). The symbiotic coral *Leptoseris fragilis* has been found at 145 m in the Red Sea, a depth to which only 0.17% of total surface irradiance penetrates (Fricke and Schuhmacher, 1983).

The ability of symbiotic zooxanthellae to tolerate extended periods of darkness is consistent with the hypothesis that zooxanthellae can survive by heterotrophic uptake of host derived organic substrates. Yonge and Nicholls (1931) reported that zooxanthellae of *Psammocora gonagra* appeared normal after 166 days of host starvation in darkness. Corals starved in darkness had 60% fewer zooxanthellae than normal corals, but these zooxanthellae were still capable of photosynthetic oxygen production (Yonge and Nicholls, 1931). Kevin and Hudson (1979) found that *Plesiastrea urvillei* maintained a normal complement of zooxanthellae during periods of total darkness for at least 48 days. Some zooxanthellae were still present after 150 days of darkness, and colonies which were bleached in darkness, then maintained in light, re-established the symbiosis (Kevin and Hudson, 1979). The recolonizing zooxanthellae were presumably derived from a population which had survived the period of darkness. Data reported here show regrowth of zooxanthellae in *Aiptasia pulchella* which had been maintained for four years in darkness, then brought into light. The reinfecting zooxanthellae were not derived from free-living populations since animals were maintained in Millipore-filtered (0.45 μ) seawater.

The results presented here suggest that laboratory maintained *A. pulchella* may incur a metabolic cost from having symbiotic algae, due to an algal requirement for heterotrophic substrates some or all of the time. However the benefit to the host from symbiosis may not lie in the photosynthetically fixed carbon which is thought to be translocated from algae to host. *In vivo* ^{31}P nuclear magnetic resonance analysis of symbiotic *A. pulchella* has shown that photosynthesis by symbiotic zooxanthellae does not significantly affect the bioenergetic status of the anemone host (Steen, 1985). Perhaps the advantage of having symbiotic zooxanthellae lies in their ability to produce oxygen in photosynthesis (Fredericks, 1976; Rinkevich and Loya, 1984), or to synthesize some organic material ordinarily limiting to the animal (Von Holt, 1968; Kokke *et al.,* 1981).

ACKNOWLEDGMENTS

Supported by a UCLA Chancellors Intern Fellowship. I thank A. Durstenfeld for performing the rat tail vein injection, and L. Muscatine, K. Dunn, J. Smith, and W. O'Loughlin for discussion and criticism.

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GENETIC DIFFERENTIATION OF LIFE HISTORY TRAITS IN POPULATIONS OF *MESOCYCLOPS EDAX* (CRUSTACEA: COPEPODA)

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ABSTRACT

Differences in maturation time, body size and clutch size among populations of the freshwater copepod *Mesocyclops edax* inhabiting lakes of different seasonalities (Lake Thonotosassa, Florida, and Douglas Lake, Michigan) were maintained through two generations under common laboratory conditions. In most cases, Florida individuals matured more rapidly and had larger body sizes and clutch sizes than Michigan individuals over a range of temperature from 15 to 30°C. The greatly reduced fecundity and longer maturation time of the Michigan population relative to the Florida population at 15°C may reflect adaptations to different temperature regimes encountered by these geographically distant populations. Genotype-environment interactions were observed for body size, but not for maturation time or clutch size. Some local variation was evident in both the Michigan and Florida locales, indicating that forces operating on a local scale may result in substantial variation that is superimposed upon any broad scale, geographic patterns.

Selection for short maturation time and large clutch size is hypothesized to have occurred in the Thonotosassa population, which is exposed to heavy fish predation and high food levels. The small bodies and clutches in the Douglas population, which experiences chronically low food levels, may reflect selection to minimize energy demands where food is scarce.

INTRODUCTION

An underlying assumption pervading much of life history theory is that at least part of the variation observed within and among populations is genetically based and represents local adaptation to specific environments. The recent growing interest in integrating ecological and genetical approaches to understand life history evolution emphasizes the fundamental need for establishing the heritable basis of such variation when investigating its adaptive significance (Dingle and Hegmann, 1982; Lande, 1982). When such experiments are conducted over a range of environments, the genetic expression of life history traits sometimes can be observed to vary according to the environment in which it is measured (Clausen *et al.,* 1948; Berven 1982a, b). Changes in relative performances across environments may alter conclusions derived from experiments conducted in just one environment, as well as provide insight into how organisms may adapt to entire environmental regimes.

The present study was designed to determine whether populations of the widely distributed freshwater zooplankter *Mesocyclops edax* (Crustacea:Copepoda) differ genetically with respect to life history traits. The isolated water bodies inhabited by freshwater copepods may reduce gene flow among populations, thereby increasing the likelihood of adaptation to specific environments. Populations inhabiting environments that differ with respect to physical and biological factors and are widely separated

Received 7 March 1985; accepted 23 January 1986.

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geographically (Florida, Michigan), were chosen to increase the probability of finding genetic differences. The life history traits examined were maturation time, adult body size, and clutch size. The responses of these traits over a temperature range of 15 to 30°C were examined to ascertain the presence of any genotype–environment interaction. Temperature is a well documented influence on the phenotypic expression of life history traits in copepods (Coker, 1933; McLaren, 1965; Burgis, 1970; Herzig, 1983), but studies that directly examine the heritable basis of such phenotypic responses to temperature are few (Battaglia, 1959; McLaren, 1976; Bradley, 1978a). In addition to temperature, food level in the study lakes is compared and its potential role as a selective force is discussed. Two populations were examined from each locale to compare the extent of local variation with large scale geographic variation.

MATERIALS AND METHODS

Study sites

The primary experimental populations were collected from Douglas Lake in Cheyboygan Co., Michigan, and Lake Thonotosassa in Hillsborough Co., Florida. One additional lake (Fairy Lake, Florida, and Lake Cochran, Michigan), each located within 35 km from the main study lake, was selected in each of the two counties to examine local variation within Michigan and Florida. Trophic classifications were based on Secchi disc transparency and chlorophyll *a* measurements and assigned to the Michigan lakes according to Carlson's (1977) trophic state index, and to the Florida lakes according to the interpretation of Carlson's index for Florida lakes by Beaver and Crisman (1982). Lake Thonotosassa is eutrophic-hypereutrophic (Cowell *et al.*, 1975; Wyngaard *et al.*, 1982) and Douglas Lake is mesotrophic-eutrophic and is described by Welch (1927), Tucker (1957) and Lind (1978). Fairy Lake, Florida, is in the upper portion of the range of trophic state indices for oligotrophic lakes (Young, 1978; Elmore, 1983) and Lake Cochran, Michigan, is mesotrophic (Gannon and Paddock, 1974; Fairchild, 1980). Lake Thonotosassa contains the highest planktivorous fish densities (Langford, 1975), followed by Douglas Lake (Smith, unpub. data), which has a diverse fish assemblage. Densities of planktivores (vertebrate and invertebrate) are low in Lake Cochran (Fairchild, 1980) and Fairy Lake (Young, 1978; Elmore, 1983). Throughout this paper individuals from Lake Thonotosassa, Fairy Lake, Lake Cochran, and Douglas Lake are referred to as FT, FF, MC, and MD individuals, respectively.

Field measurements

Copepods were collected with a No. 20 net (76 μm), narcotized with CO_2 and preserved in 4% formaldehyde before measuring body size. Female body size and clutch size of MD and FT individuals were measured several times during the year to span a range of temperatures. Egg volumes were measured using MD and FT individuals collected during early summer. Egg volume was determined by measuring five eggs from each clutch, and calculated using the volume of a prolate spheroid $= 4/3\ r_1 r_2^2$, where r_1 is the long axis and r_2 is the short axis. Male body size of MD and FT individuals was measured using collections from May and September, respectively, to obtain males that matured at comparable temperatures. Cephalothorax length was measured at 40× with a compound microscope. Body size and clutch size of MD and FT females were compared using samples collected during several seasons. Body size and clutch size of FF individuals were determined from a spring and summer collection and those of the MC population from a summer collection.

Chlorophyll *a* concentrations, used as a measure of food level, were determined from samples collected bi-weekly from Douglas Lake from June through October, 1980 in the deepest portion of South Fishtail Bay and weekly measurements at the surface, 5 m, 10 m, 15 m, and 17 m from 3 July to 28 July (Evans and Glover, unpub. data) also were available. Chlorophyll samples from Lake Cochran were collected at monthly intervals at 1.5 m from June–September, 1980, in the deepest portion of the lake. The procedures used to measure chlorophyll *a* content of water samples from the Michigan lakes were the same as those used to analyze the Florida water samples (Wyngaard *et al.*, 1982; Elmore, 1980), except that a Turner Fluorometer III was used in the former analyses.

Laboratory rearing

The life cycle of *M. edax* consists of an embryonic (egg) stage, six larval (nauplius) stages, five juvenile (copepodid) stages, and an adult stage which does not molt. Michigan populations show a facultative diapause from late fall to early spring. From spring to fall the Michigan populations complete two generations without any diapause. In the Florida population development from egg to adult proceeds continuously throughout the year. The typical diet during the early stages consists of algae, protozoans, and bacteria and diversifies to include larger animals (rotifers, cladocerans, copepods) in the later stages.

For the laboratory rearing studies ovigerous females were collected from the field and their eggs allowed to hatch under the experimental conditions. The only exceptions to this were the 25°C life tables of FT and MD populations for which virgin females were mated in the laboratory. Animals were reared individually in the laboratory for two generations under constant, defined conditions to ensure that all populations were reared in a common environment and that conditions were repeatable over time. Experiments were generally initiated when the lake temperature approximated the laboratory temperature (Table I). MD and FT copepods were reared at 15 ± 0.5°C, 25 ± 0.5°C and 30 ± 0.5°C to determine expression of life history traits at each of three temperatures. FF and MC individuals were reared at 25 ± 0.5°C for examination of local variation. All collections of Michigan animals were made in early summer to ensure that laboratory generations one and two paralleled those in the field and did not overlap with the period when animals normally were in diapause.

TABLE I

Collection dates and lake temperatures of laboratory reared populations of Mesocyclops edax

Population	Date collected	Lake temperature °C	Laboratory temperature °C
Michigan			
Douglas	June 1982	21	15
Douglas	June 1981	22	25
Douglas	June 1982	21	30
Cochran	June 1982	22	25
Florida			
Thonotosassa[1]	February 1980	12.8	15
Thonotosassa	July 1981	31.0	25
Thonotosassa	August 1982 (rep 1)	28.8	30
	June 1982 (rep 2)	28.0	25
Fairy	April 1982 (rep 1)	24.4	25
	September 1982 (rep 2)	29.5	25

Life tables of the FT individuals reared at 30°C and FF individuals reared at 25°C were replicated with samples collected during different seasons in order to reveal genetically based seasonal variation that may have existed within a population (Table I).

Upon hatching, nauplii were transferred to a petri dish 20 mm in depth and 60 mm in diameter containing 15 ml of artificial lake water and 10^4 cells/ml of the flagellated yellow-brown alga *Cryptomonas ozolini* Sküjja. Individuals were transferred by a wide pipette every other day to a clean dish containing fresh medium and algae. Handling mortality was clearly negligible, as survival at 25°C was 97%. Food densities did not decrease below 10^4 cells/ml on the second day. Upon reaching maturity, adults were fed three juveniles (copepodid stages I–III) of the calanoid copepod *Diaptomus dorsalis* (Marsh) daily. The light:dark cycle for all organisms reared in this study was 14:10.

C. ozolini was isolated from Douglas Lake by D. Fuller and can be obtained from the Starr Collection at the University of Texas at Austin (culture number UTEX-LB2194). Formulae for the artificial lake water and algal media and details of culture methods of alga and *Diaptomus* (from Lake Thonotosassa) are given in Wyngaard and Chinnappa (1982).

Because of very low reproduction of the MD population at 15°C, only one generation was reared at this temperature. The trends in statistically significant differences between the FT, FF, and MC populations were similar for both generations (Wyngaard, 1983); thus the analyses of the four populations presented in this paper were based upon the first generation only.

Statistical procedures

Statistical tests used in this study included *t*-tests and 2 × 2 tests of independence (*G*-statistic) (Sokal and Rohlf, 1969). Statistical significance is taken to be .05 or less. The experimental design for examining genotype-environment interactions yielded unbalanced data sets, which were analyzed using the GLM procedure of S.A.S. (Helwig and Council, 1979). Sample sizes used in the analyses for any one trait ranged from 474–707 individuals. As some individuals were related (*i.e.*, brothers or sisters of same brood), the ANOVA were performed on family means that were weighted by family size, yielding 93–225 observations. Family size ranged from one to ten individuals. Least squares means were calculated for all laboratory reared animals in this study, so that large and small families would be weighted accordingly. One trait, maturation time, was transformed to natural logarithms. The assumption of homogeneity of variances among treatments was satisfied in all tests.

RESULTS

Field analyses

Field collections revealed differences between the MD and FT populations in most traits examined (Table II). Similar trends between populations in female body size and clutch size occurred regardless of season, with the exception of FT females collected in winter which were smaller than MD females collected during spring and summer (Appendix). Thus samples collected during several seasons over a year were lumped in the comparisons. FT females were significantly larger and had larger clutches, but significantly smaller eggs than MD females (Table II). FT males were larger than MD males. The coefficients of variation for specific traits were similar between populations,

TABLE II

Means ($\overline{X}$) and coefficients of variation (C.V.) of traits of field caught Mesocyclops edax *from Douglas Lake and Lake Cochran, Michigan, and Lake Thonotosassa and Fairy Lake, Florida*

	Michigan				Florida				
	Douglas		Cochran		Thonotosassa		Fairy		
Trait	$\overline{X}$	(C.V.)	$\overline{X}$	(C.V.)	$\overline{X}$	(C.V.)	$\overline{X}$	(C.V.)	*P* (MD *vs.* FT)
Female body size (μm)	696.0 n = 138	(9.5)	718.1 n = 89	(7.0)	761.9 n = 164	(6.8)	758.3 n = 126	(6.3)	$P < .001$
Male body size (μm)	416.2 n = 50	(3.8)			491.0 n = 52	(4.7)			$P < .001$
Clutch size	20.4 n = 50	(16.1)	22.4 n = 20	(14.1)	51.2 n = 154	(29.2)	33.5 n = 34	(23.9)	$P < .001$
Egg volume ($mm^3 \times 10^{-3}$)	0.571 n = 70	(23.3)			0.239 n = 70	(32.2)			$P < .001$

Numbers of individuals are indicated by n. Means of populations are compared using *t*-tests.

with the exception of clutch size which was markedly less variable in the MD population (16.1%) than in the FT population (29.2%).

Samples of Michigan copepods collected in June most likely contained individuals that experienced comparable temperatures throughout their lifetime, as they in all likelihood hatched from eggs produced by the previous diapausing generation and were used to compare field collected MD and MC individuals. MD females (737.8 μm) were larger than MC females (718.1 μm) (t(144 df) = 2.2, $P < .01$), but mean clutch size of MD females (20.06) was smaller (t(49 df) = 24, $P < .05$) than MC females (22.45). Female sizes were not significantly different between FT and FF populations averaged over the year, but FT females had larger clutches than FF females (t′(186 df) = 9.68, $P < .001$) (Table II). Clearly individuals from lakes within a locale were more similar than those between Michigan and Florida.

Laboratory analyses

Maturation time. FT females and males matured significantly more rapidly than MD individuals when reared in a common laboratory environment at 15, 25, and 30°C, except for males which did not differ significantly at 30°C (Fig. 1). Females and males showed similar responses over the temperature range as evidenced by the lack of a population–environment interaction.

Local variation was evident at both locales (Table IV). MD females matured significantly more rapidly (15.8 days) than did MC females (20.6 days). Both FT females and males matured more rapidly than FF females and males, respectively.

Body size. FT females and males had larger body sizes than MD females and males, respectively, at 15 and 25°C (Fig. 2). FT and MD females did not differ significantly at 30°C but MD males were significantly larger than FT males. Generation two FT and MD males were used in the comparison at 25°C because many MD males of generation one at this temperature were eaten by females following mating, and could not be measured. This predation may have resulted in a biased sample of males available for measurement, and could explain why mean body size of generation one

TABLE III

ANOVA for maturation time in two populations (Douglas and Thonotosassa) of Mesocyclops edax *in three environments (15, 25, and 30°C)*

Females

Source	d.f.	M.S.	F	*P*
Population	1	0.727	23.6	<.001
Environment	2	33.954	1103.9	<.001
Population × environment	2	0.016	0.5	N.S.
Error	220	0.031		
Total	225			

Males

Source	d.f.	M.S.	F	*P*
Population	1	0.251	10.6	<.001
Environment	2	31.558	330.7	<.001
Population × environment	2	0.052	2.2	N.S.
Error	186	0.024		
Total	191			

FT males (466.3 μm) reared at 25°C was less than the mean body size of MD males (482.7 μm) (Wyngaard, 1983b). For this reason when rearing generation two at 25°C, randomly selected groups of FT and MD males were not mated but instead preserved upon reaching maturity. Generation two FT males reared at 25°C were significantly larger than generation two MD males (t′(136 df) = 3.95, $P < .001$) (Fig. 2). These latter results more likely reflect the true comparison of male body size of the FT and MD populations. Difference in body size was not evident between populations at the

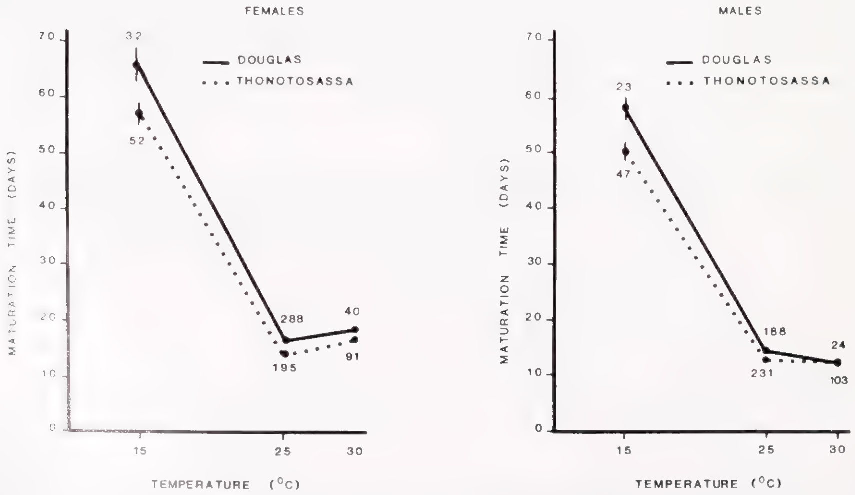

FIGURE 1. Mean maturation time of female and male *Mesocyclops edax* at three temperatures from Douglas Lake, Michigan (——) and Lake Thonotosassa, Florida (– – –). Numbers and vertical lines refer to number of individuals and one standard error, respectively. Error bars at 25 to 30°C are contained within the dot.

TABLE IV

Least squares means ($\overline{X}$) and standard errors (S.E.) of life history traits of generation one Lake Thonotosassa and Fairy Lake, Florida, and Douglas Lake and Lake Cochran, Michigan, individuals reared at 25°C

	Florida					Michigan				
	Thonotosassa		Fairy			Douglas		Cochran		
	$\overline{X}$	(S.E.)	$\overline{X}$	(S.E.)	*P*	$\overline{X}$	(S.E.)	$\overline{X}$	(S.E.)	*P*
Females										
Maturation time (days)	14.0	(+0.39) (−0.38)	18.1	(+0.05) (−0.05)	<.001	15.7	(+0.39) (−0.38)	20.5	(+0.47) (−0.46)	<.001
	n = 195		n = 71			n = 188		n = 62		
Body size (μm)	699.3	(6.17)	649.3	(7.31)	<.001	663.3	(5.16)	679.2	(7.68)	N.S.
	n = 144		n = 63			n = 226		n = 45		
Clutch size	35.51	(0.93)	24.20	(0.79)	<.001	21.95	(0.82)	19.90	(1.03)	N.S.
	n = 59		n = 30			n = 70		n = 19		
Males										
Maturation time (days)	12.5	(+0.32) (−0.30)	14.0	(+0.29) (−0.28)	<.01	13.6	(+0.35) (−0.34)	14.2	(+0.25) (−0.25)	N.S.
	n = 231		n = 52			n = 188		n = 63		
Body size (μm)	499.6	(4.26)	475.1	(4.95)	<.01	482.7	(5.06)	491.2	(3.70)	N.S.
	n = 92		n = 29			n = 36		n = 59		
Survival	96.77		84.82		<.005	97.16		76.68		<.001

Geometric means and 95% confidence intervals of maturation time are presented, as these data were log transformed in the statistical analyses. Numbers of individuals are indicated by n. Probability limits are for comparisons between populations within each locale.

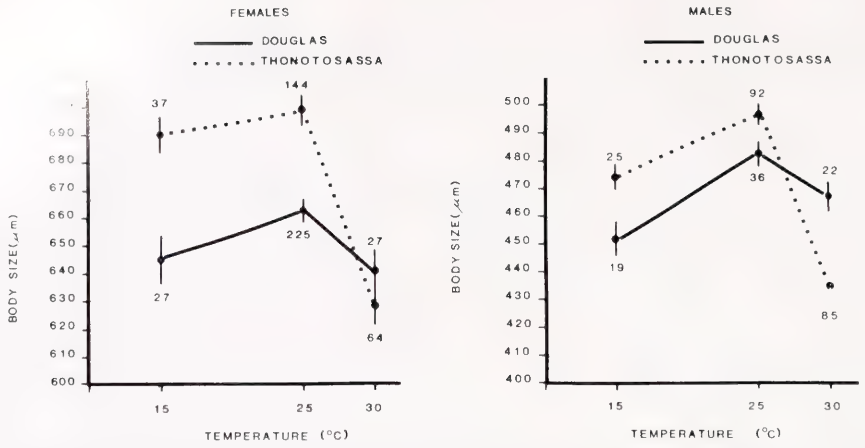

FIGURE 2. Mean body size of female and male *Mesocyclops edax* at three temperatures from Douglas Lake, Michigan (——) and Lake Thonotosassa, Florida (- - -). Numbers and vertical lines refer to number of individuals and one standard error, respectively.

Michigan locale, but FT females and males were larger than FF females and males, respectively (Table IV).

Female and male body sizes were the only traits in this study that exhibited a significant genotype-environment interaction, as examined by two-way ANOVA ($F_{2,189} = 10.09$, $P < .001$ for females; $F_{2,135} = 15.00$, $P < .001$ for males) (Table V) and response curves (Fig. 2). Males were significantly smaller at 15°C than at 25°C in both MD (t(53 df) = 3.70, $P < .001$) and FT (t′(115 df) = 3.96, $P < .001$) populations.

Clutch size. FT individuals had larger clutch sizes than MD individuals at 15, 25, and 30°C (Fig. 3). Only one MD female of generation one produced a clutch at 15°C. This clutch had 19 eggs, which is typical for this population at all temperatures in the

TABLE V

ANOVA of adult body (μm) size in two populations (Douglas and Thonotosassa) of Mesocyclops edax *in three environments (15, 25 and 30°C)*

Females

Source	d.f.	M.S.	F	*P*
Population	1	2343000	17.6	<.001
Environment	2	3937000	29.5	<.001
Population × environment	2	1344000	10.1	<.001
Error	189	133000		
Total	194			

Males

Source	d.f.	M.S.	F	*P*
Population	1	220000	4.9	<.05
Environment	2	613000	13.5	<.001
Population × environment	2	680000	15.0	<.001
Error	135	45000		
Total	140			

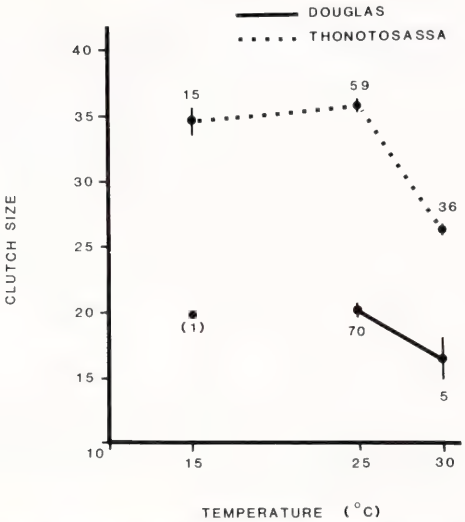

FIGURE 3. Mean clutch size of female *Mesocyclops edax* at three temperatures from Douglas Lake, Michigan (——) and Lake Thonotosassa, Florida (- - -). Numbers and vertical lines refer to number of individuals and one standard error, respectively.

field and lies outside the 95% confidence limits for the average clutch size (35.5) in the FT population at 15°C. Of the females offered males for mating, only one of 18 (5%) MD females and 15 of 48 (31%) FT females produced clutches. FT females had larger clutches than FF females, whereas clutch size was not significantly different between populations at the Michigan locale (Table IV).

Survival. Survival from hatching to adult varied considerably across temperatures. Both MD and FT individuals had highest survival (97.2%, 96.7%, respectively) at 25°C, and moderate survival (51.6%, 52.6%, respectively) at 30°C. A statistically significant difference in survival between these two populations was evident only at 15°C, at which FT individuals survivied almost twice as well (64.7%) as MD individuals (36.7%) (G(1 df) = 24.14, $P < .005$). Survival was significantly higher (97.2%) in the MD population than in the MC population (76.7%) and significantly higher in the FT population (96.8%) than the FF population (84.8%). Survival of FF and MC individuals was not significantly different.

Seasonal variation and acclimation. Additional life tables compared copepods from FT and FF populations collected at different seasons and reared through two generations in the laboratory. These studies revealed that the genetically based seasonal variation

TABLE VI

ANOVA of clutch size in two populations (Douglas and Thonotosassa) of Mesocyclops edax *in two environments (25 and 30°C)*

Females

Source	d.f.	M.S.	F	*P*
Population	1	1766	101.8	<.001
Environment	1	677	39.0	<.001
Population × environment	1	53	3.1	N.S.
Error	90	17		
Total	93			

was minimal and did not alter the trends reported above. Mean values for FT individuals at 30°C reared from June and August collections were similar (Table VII), as were those of FF individuals reared at 25°C from May and September collections (Table VIII). In the instances in which statistically significant differences in mean values between replicate life tables exist, the differences between either replicate and the MD or FT population was in the same direction as reported in the above analyses. For example, comparison of FT individuals reared at 30°C from either the June or September collections to MD individuals reared at 30°C revealed the same trends in statistically significant differences. The only exception was a nonsignificant difference in female maturation time between MD and FT females collected in June. However, maturation time was not significantly different between the FT females reared from the September and June collections.

The sample sizes for generation one were larger than those of generation two in most cases, but the trends in statistically significant differences in life history traits between the MD *versus* MC and MD *versus* FT populations were the same for both generations (Wyngaard 1983). Exceptions to this occurred at 25°C at which generation one MD males were larger than FT males (see above) and at 30°C at which generation two FT males matured significantly faster than MD males.

The rearings of all FF populations were notable in that sex ratios were strongly biased toward females. This sex bias in the generation two FF population resulted in such a small number of males that it is difficult to ascribe any trends to certain traits

TABLE VII

Least squares means ($\overline{X}$) and standard errors (S.E.) of generation one individuals reared at 30°C in 1982 from June and August collections from Lake Thonotosassa, Florida, and a June collection from Douglas Lake, Michigan

	Douglas June			Thonotosassa June			Thonotosassa August	
	$\overline{X}$	(S.E.)		$\overline{X}$	(S.E.)		$\overline{X}$	(S.E.)
Females								
Maturation time	17.3	(+0.55) (−0.54)		16.6	(+0.49) (−0.47)		15.8	(+0.38) (−0.37)
	n = 40			n = 50			n = 91	
			N.S.			N.S.		
Body size (μm)	640.1	(7.61)		612.1	(5.63)		628.7	(5.44)
	n = 27			n = 37			n = 64	
			$P < .01$			$P < .05$		
Clutch size	16.80	(2.18)		24.9	(2.42)		26.4	(0.93)
	n = 5			n = 9			n = 38	
			$P < .001$			N.S.		
Males								
Maturation time	12.4	(+0.48) (−0.45)		13.18	(+0.36) (−0.36)		12.2	(+0.26) (−0.25)
	n = 24			n = 48			n = 103	
			N.S.			$P < .05$		
Body size (μm)	467.1	(5.32)		425.1	(5.22)		435.3	(3.01)
	n = 22			n = 20			n = 85	
			$P < .001$			N.S.		
% Survival	51.61			50.00			52.57	
			N.S.			N.S.		

Geometric means and 95% confidence intervals of maturation time are presented, as these data were log transformed in the statistical analyses. Number of individuals is indicated by n. Means of populations are compared using *t*-tests.

TABLE VIII

Least squares means ($\overline{X}$) and standard errors (S.E.) of generation one individuals reared at 25°C from collections in May and September of 1982 from Fairy Lake, Florida, and summer of 1981 from Lake Thonotosassa, Florida

Trait	Thonotosassa July $\overline{X}$	(S.E.)		Fairy September $\overline{X}$	(S.E.)		Fairy May $\overline{X}$	(S.E.)
Females								
Maturation time	14.0	(+0.39) (−0.38)		22.9	(+0.80) (−0.77)		18.1	(+0.05) (−0.06)
	n = 195		$P < .001$	n = 59		$P < .001$	n = 71	
Body size (μm)	699.3	(6.17)		630.4	(5.93)		649.3	(7.31)
	n = 59		$P < .001$	n = 46		$P < .05$	n = 63	
Clutch size	35.51	(0.93)		24.25	(1.84)		24.20	(0.79)
	n = 59		$P < .001$	n = 4		N.S.	n = 30	
Males								
Maturation time	12.5	(+0.32) (−0.30)		20.1	(+1.43) (−1.12)		14.0	(+0.29) (−0.28)
	n = 231		$P < .001$	n = 14		$P < .001$	n = 52	
Body size (μm)	499.6	(4.26)		439.1	(5.86)		475.1	(4.95)
	n = 92		$P < .001$	n = 12		$P < .001$	n = 29	
% Survival	96.77			51.02			84.82	
			$P < .001$			$P < .001$		

Geometric means and 95% confidence intervals of maturation time are presented as these data were log transformed in the statistical analyses. Numbers of individuals are indicated by n. Means of populations are compared using *t*-tests.

with statistical confidence. Nevertheless generation one individuals did not show more statistically significant differences in traits among the four populations than generation two individuals, indicating that acclimation effects were minimal.

Summary. In most cases FT individuals matured significantly more rapidly, and had larger body sizes and clutch sizes than MD individuals when reared in a common laboratory environment at 15, 25, and 30°C (Figs. 1–3). Only female and male sizes exhibited a genotype-environment interaction. Local variation was evident in some life history traits at both the Michigan and Florida locales, but was greater at the Florida locale (Table V). These trends between populations did not change over the seasons (Tables VII, VIII).

Trophic characteristics of lakes

The four study lakes can be assigned to low or high food level, as measured by chlorophyll *a* content and algal cell volume (Table IX). Lake Thonotosassa exhibited by far the highest values; Douglas Lake, Lake Cochran, and Fairy Lake values were somewhat lower.

DISCUSSION

This study documents genetic differentiation with respect to several life history traits in geographically distinct as well as in local populations of the copepod *Mesocyclops edax.* The Michigan and Florida populations differed in all life history traits

TABLE IX

[illegible] the Michigan and Florida study lakes that reflect food level

	Florida		Michigan	
	Fairy	Thonotosassa	Douglas	Cochran
algal cell volume (μl/l)	3.9[a]	11.9[a]	3.5[a]	—
chlorophyll *a* (μl/l)	10.9[a]	91.6[a]	6.0[d,c]	3.0[b,f]
(range)	(1.9–31.6)[a,c,f]	(26.0–110.4)[a,c,e]	(1.5–14.5)[b,d,f]	(0.8–11.0)[b,f]
percent blue green algae	18[a]	68[a]	80[b]	—
percent green algae	78[a]	26[a]	3[b]	

[a] Dawes and Cowell (1984).
[b] Fairchild (1980).
[c] Elmore (1983).
[d] Evans and Glover (unpub. data).
[e] Wyngaard *et al.* (1982).
[f] Wyngaard (unpub. data).

examined. FT individuals in most cases had the largest adult body and clutch sizes coupled with the most rapid maturation. MD and MC individuals were smaller, had smaller clutches, and slower maturation, whereas FF individuals were rougly intermediate between the Michigan populations and their FT counterparts. The genetically based differences in maturation time, body size, and clutch size among populations of *M. edax* suggest these populations have travelled separate evolutionary paths during the recent past. Additional genetical studies at another level support this view; the additive genetic variances in maturation time, body size, and clutch size also differ between the Michigan and Florida populations (Wyngaard, 1986). This species is one of the most widely distributed freshwater cyclopoids in North America, lives in water bodies of various sizes, trophy, pH, and community composition, and exhibits considerable phenotypic variation among populations (Forbes, 1897; Coker, 1943; Wilson and Yeatman, 1959; Wyngaard and Chinnappa, 1982). The present study clearly reveals that some of this phenotypic variation is genetically, rather than environmentally based. It is still premature, however, to conclude whether these differences reflect large scale geographic patterns or only local variation.

The relative magnitude of local compared to large scale geographic variation may depend, in part, upon barriers to gene flow. Strong genetic differentiation in the face of high levels of gene flow has been documented (Jain and Bradshaw, 1966; Antonovics and Bradshaw, 1970; Endler, 1973; McNeilly, 1968). Allan (1984), however, found no evidence of reproductive isolation between the MD and FT populations.

Possible explanations for genetic differences observed in the present study are founder effect, random drift, and different selective regimes. The wide geographical distribution and characteristically large population sizes of *M. edax* suggest that the first two explanations are not probable causes of the observed genetic differentiation. Studies of rates of gene flow, however, are necessary to rule out these two explanations.

The role of selection in molding the observed life histories is a logical consideration as these populations have presumably evolved in lakes that differ in temperature regime, resource availability, and predator level. Numerous ecological studies have implicated these factors as ones influencing population dynamics and phenotypic differences in life histories (review in Kerfoot, 1980).

Role of temperature

Two lines of thought suggest that the MD population is adapted to a narrower temperature range than the FT population. First, body size of the MD population changes little over the temperature range examined in this study relative to that of the FT population. Body size may change little, or be directly related to temperature, outside an optimal temperature range, as reported for hemimetabolous aquatic insects (Sweeny and Vannote, 1978). Second, MD individuals occur in non-diapausing states over the temperature range of about 12–27°C, but the greater portion of their development and reproduction in the lake occurs between 20–26°C. In contrast, FT individuals may develop through all stages and reproduce in the lake over a temperature range of about 12–31°C, though reproduction nearly ceases between 12–15°C (Wyngaard *et al.*, 1982). *Mesocyclops* is subtropical in origin (Rylov, 1948) and 15°C probably represents a lower thermal boundary for development.

The near cessation of reproduction by the MD adult females at 15°C is a striking contrast to the fecund FT population. The MD females reared at 15°C certainly were not in diapause, as they actively swam and consumed live *Diaptomus* prey as well as males of *M. edax* that were presented to them for mating purposes. Diapause in *M. edax* is usually characterized by arrested development at the fourth copepodid stage, accumulation of lipids, presence of mudcaps on either side of the cephalothorax, and a quiescent period during which the animals sink to the bottom muds during the fall and remain there until they emerge into the water column the following spring (Comita, 1972; Stucke, 1981). Diapause of adult females has also been reported in a Connecticut population (Elgmork, Univ. of Oslo, pers. comm.). It is possible that insemination occurred, but that 15°C was too cold for fertilization or production of egg sacs. Low temperatures prevent inseminated females of the marine copepod *Acartia tonsa* from producing egg sacs (Lonsdale, SUNY, Stony Brook, pers. comm.).

Genetic differentiation with regard to temperature has been suggested to be more prevalent in stenothermal species or races; acclimation, in eurythermal species or races (Precht *et al.*, 1973). Herzig's suggestion that the relative importance of genetic differentiation and acclimation may shift according to the temperature and geographic ranges experienced by the particular zooplankton species seems plausible in the context of the present study. He suggested that the more rapid development of *M. edax* eggs of an Ontario pond population (Carter, 1974) relative to a Lake Ontario population (Cooley, 1978) reflected adaptation to different temperature regimes. *M. edax* is a warm water copepod and Herzig's comparisons, as well as those in the present study, seem consistent with the view that genetic adaptation (or genetic differentiation) rather than acclimation is responsible for much of the phenotypic variation observed in field populations of *M. edax*. The ground water species *Speocyclops gallicus* is an extreme case of a copepod that experiences nearly constant temperature conditions, and shows a great degree of genetic adaptation in temperature related traits such as egg stage duration (Lescher-Moutoue, 1973). In contrast to the temperature responses of stenothermal copepods, the estuarine eurythermal copepod *Eurytemora affinis*, which typically experiences a temperature range of 0–30°C, shows considerable acclimation (Bradley, 1978a, b). The different measurement techniques used by different investigators may lead to a potential difficulty in distinguishing adaptation from acclimation in these studies. The environmental temperatures experienced by both populations in the present study, however, do not help explain why the FT individuals attained greater size or matured more rapidly than did MD individuals at an intermediate temperature (25°C).

Confounding selection pressures likely contribute to the complexity of these life histories and the difficulties of attributing the observed differences among populations to one factor or another. In addition to temperature, abundance of food and predators has been measured in the study lakes (cited below) and received considerable attention in the zooplankton literature as potential selective forces that influence life histories. Thus, it seems appropriate to comment on the possible relevance of differences in food level and predator abundance among the study lakes to the genetic differentiation observed in this study.

Role of food level

The two northern sites did not differ greatly from one another in any obvious factors such as trophic status and, probably for that reason, exhibited no genetic differentiation except for one trait, female maturation time. The Florida locale afforded an opportunity to examine two lakes that are situated in close proximity but have grossly different trophic status (Wyngaard *et al.,* 1982; Dawes and Cowell, 1984; present study) and community structure (Elmore, 1983) in the same geographical region. For this reason, genetic differentiation may be greater between the two Florida populations. Elmore (1983) showed that the high and low food levels in Lake Thonotosassa and Fairy Lake, respectively, coupled with differences in predator abundance, constitute different selective regimes for three diaptomid copepod species. Food and predation determined, in large part, their distribution and life history characteristics.

The small body size, slow maturation, small clutches, and large eggs of the Michigan populations may be viewed as consequences of selection to minimize energy demands when food is scarce. *In situ* culture of MD and FT individuals in (low-food) Douglas Lake revealed that MD individuals hatched from larger eggs into larger nauplii and exhibited higher survival to maturity than did FT individuals (Wyngaard, unpub. ms.). Allan (1984) found this difference in egg size to be genetic and reported a negative phenotypic correlation between egg size and clutch size when the MD and FT populations were pooled, suggesting a tradeoff between the traits of egg size and clutch size.

Role of predation

Lake Thonotosassa contains high densities of planktivorous fishes, the most abundant of which is gizzard shad (Langford, 1975). Fairy Lake contains no shad. FT individuals undergo a pronounced diel vertical migration (Wyngaard, 1978) whereas the FF individuals do not do so. This is consistent with the view that vertical migration behavior is a response to the feeding behavior of fish (Zaret and Suffern, 1976). It has been argued that predation selects for rapid maturation (Charlesworth, 1980) and small size at first reproduction (Brooks and Dodson, 1965). The rapid maturation of FT individuals may be a response to such predation pressure. The large body sizes of the FT individuals may seem inconsistent with this view, but gizzard shad in Lake Thonotosassa likely feed upon zooplankton using a pump filter mechanism rather than visual attack on plankton (Drenner *et al.,* 1982).

While the Michigan lakes contain planktivorous fishes (Fairchild, 1980; Smith, American Museum of Natural History, unpub. data) the higher densities of shad in Lake Thonotosossa probably represent a level of predation much greater than that of Lake Cochran or Douglas Lake. Fairy Lake and the two Michigan lakes contain high densities of daphnids (Young, 1978; Fairchild, 1980; Dawes and Cowell, 1984; Gannon, SUNY at Oswego, unpub. data), which are not typically abundant in lakes with high densities of planktivorous fish. The low food levels in these three lakes may exhibit more influence on life history characteristics than predation, resulting in small body sizes and slow maturation. The interplay among temperature, predation, and resource availability is difficult to dissect. The present study hints at what might be the relative importance to life histories of these factors in specific populations.

These interpretations of genetic differentiation among the four *M. edax* populations represent hypotheses describing how different environments may have selected for different life histories. Perhaps what is most intriguing is the observation of two seemingly distinct life history tactics: (1) rapid maturation, large body, large clutches, and small eggs in the Florida population and (2) slow maturation, small body, small clutches, and large eggs in the Michigan population. It would be of interest to investigate whether these assemblages of traits represent tightly, co-adapted complexes of life history traits, or alternatively, independently evolving individual traits.

ACKNOWLEDGMENTS

I am grateful to K. Berven, B. Bradley, J. Coyne, D. Gill, J. Elmore, C. Istock, D. Policansky, D. Reznick, and W. Walton, for stimulating discussion or helpful comments on the manuscript. J. D. Allan provided inspiration, encouragement, support, and cooperation throughout all aspects of this study. I thank R. Glover and M. Evans for providing me with their unpublished data on phytoplankton cell volume counts and chlorophyll *a* concentrations. R. Stemberger provided the algae used to culture the copepods and much appreciated expertise in algal culture. I thank J. Beechum, L. Friedman, T. McTigue, and S. Wilson for their careful assistance in the laboratory. I appreciate the advice L. Douglass, B. Mock, and E. Russek gave for statistical and computer analyses. B. Cowell and C. Hull graciously collected and shipped plankton. The University of Michigan Biological Station and University of South Florida provided field equipment and facilities to collect the field samples. This work was supported by NSF-DEB80-04450 and University of Maryland Computer Services grant to J. D. Allan and a Sigma Xi Grant-in-Aid for Research to the author.

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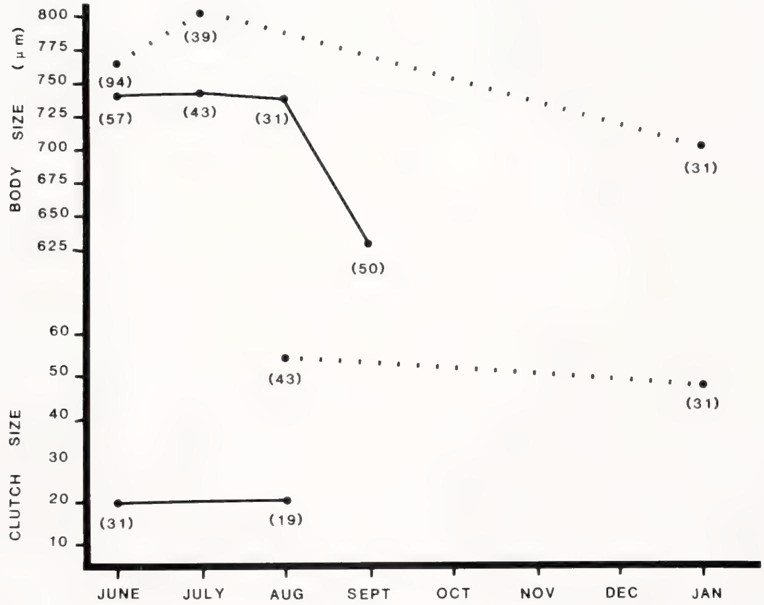

Appendix. Body size (μm) and clutch size of *Mesocyclops edax* collected from Douglas Lake, Michigan (——) and Lake Thonotosassa, Florida (- - -). Numbers in parentheses refer to numbers of individuals.

Reference: *Biol. Bull.* **170:** 296–304. (April, 1986)

HERITABLE LIFE HISTORY VARIATION IN WIDELY SEPARATED POPULATIONS OF *MESOCYCLOPS EDAX* (CRUSTACEA: COPEPODA)

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ABSTRACT

Additive genetic variances were estimated for several life history traits of north temperate Michigan and subtropical Florida populations of the freshwater copepod *Mesocyclops edax* (Crustacea: Copepoda). Estimates were obtained by rearing animals at 25°C and using a paternal half sib design. Considerable additive genetic variance was revealed in female and male body sizes of the Florida population and in female and male maturation times in the Michigan population. Female maturation time and clutch size in the Florida population and female body size and clutch size in the Michigan population exhibited low additive genetic variance. These findings provide further evidence of substantial additive genetic variance in life history traits of natural populations. The contrasting trends in additive genetic variance underlying life history traits of these populations are interpreted as reflecting differences in the potential to respond to selection and possibly also the different selective regimes of the Michigan and Florida environments.

INTRODUCTION

The variation in many life history traits of interest to evolutionary biologists is polygenic (Istock, 1981; Dingle and Hegmann, 1982; Lande, 1982) and suitable for quantitative genetic analyses. Plant and animal breeders have used such analyses to partition phenotypic variation into genetic and environmental components, and thus to predict responses to selection. Much experimental work on domesticated animals and plants, and on laboratory *Drosophila* populations, has documented changes in the proportion of additive genetic variance relative to other sources of variation after selection has been applied (see Falconer, 1980, for review). Often these findings bear out the predictions of Fishers' Fundamental Theorem of Natural Selection (Fisher, 1958): that traits exhibiting low additive genetic variance and thus showing low heritability (ratio of additive genetic variance to total phenotypic variance) are presumed to have had much of their additive genetic variance trimmed away by directional selection. It is not known, however, whether this principle can be generally applied to natural populations.

Some recent studies have uncovered substantial heritable variation in life history traits in natural populations and have attributed this variation to frequent changes in direction of selection (Dingle *et al.*, 1977; Istock, 1981) or to weak selection and negative pleiotropy (Berven and Gill, 1983). As a population's potential response to selection is, in theory, proportional to the heritable variation in fitness, the additive genetic variances of fitness characters are of interest when investigating life history evolution. If adaptation to a new environment results in changes in the amounts of additive genetic variance of particular traits, then changes in additive genetic variance

Received 7 March 1985; accepted 23 January 1986.

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might be useful for inferring shifts in the relative contributions of traits to fitness. This line of reasoning was first applied to heritability analyses of a marine copepod by McLaren (1976) and of an estuarine copepod by Bradley (1978). Few organisms evolve rapidly in nature to provide an opportunity to observe such changes in additive genetic variance. An alternative is to examine geographically isolated populations of the same species that have presumably undergone evolution and genetic differentiation in response to their separate environments. This comparative approach attempts to provide insight into why specific traits might exhibit high additive genetic variance in one population and low additive genetic variance in another.

In this study I estimate the additive genetic variances underlying phenotypic variation in several life history traits in two populations of *Mesocyclops edax* (S. A. Forbes) (Crustacea:Copepoda) from different locales. The two environments differ in several obvious ways: trophic status, thermal regime, and predator abundance and diversity. These populations show genetically based differences in the means of several traits (Wyngaard, 1986). Individuals from Florida exhibit more rapid maturation rates, larger body sizes, and larger clutch sizes than do those from Michigan. These traits are known to influence either survival and fecundity in natural zooplankton populations (Allan, 1976; Lynch, 1980; Kerfoot, 1980) and thus may be viewed as important fitness components. These two populations show no reproductive barriers in the laboratory, have fertile hybrids (Allan, 1984), and thus seem to be conspecific. The genetic differentiation in life history traits between the populations leads one to ask whether there have also been changes in the heritable variation of these traits. The purpose of this study was to estimate and compare the additive genetic variances of several fitness components among two geographically distinct copepod populations and to explore the usefulness of this information in making inferences regarding the potential to respond to selection as well as the past selective regimes experienced by these populations.

MATERIALS AND METHODS

Field study sites

Individuals of *Mesocyclops edax* were collected from Lake Thonotosassa, Hillsborough County, Florida, on 10 July 1981 and from Douglas Lake, Cheyboygan County, Michigan, on 23–26 June 1981. Detailed descriptions of Lake Thonotosassa are given by Cowell *et al.* (1975) and Wyngaard *et al.* (1982) and descriptions of Douglas Lake are provided by Welch (1927), Tucker (1957), and Lind (1978). Here the Lake Thonotosassa population is designated as FT and the Douglas Lake population as MD. Plankton were shipped by air to the laboratory within 24 h of collection.

Experimental design

Additive genetic variance components and narrow sense heritabilities were estimated with a paternal half sib mating design (*e.g.*, Falconer, 1980), using laboratory-reared animals that had undergone one generation of laboratory acclimation. Haphazardly sampled adult males and juvenile females from each population in nature were isolated in pairs and mated to yield the acclimation generation (G_1). Sixty-nine MD (Douglas Lake, Michigan) and sixty-six FT (Lake Thonotosassa, Florida) matings were successful; four to twenty progeny from each of these matings were reared to adulthood. The adults of this acclimation generation were mated using a paternal half sib mating design in which two females (dams) were mated to a single male (sire). No adult was related to any other adult in the mating scheme. A total of 14 MD and 15 FT half sib families were obtained. Twenty offspring from each dam were reared to maturity and mated, taking care to mate only unrelated individuals. These offspring comprised the second generation (G_2) on which the additive genetic variances of the life history traits were estimated.

No acclimation effects in maturation rate, body size (cephalothorax length), and clutch size were revealed between the two generations reared in the laboratory as mean values of these traits did not differ significantly (Wyngaard, 1983). Survival from hatching to adulthood of G_1 individuals in both populations was 97%. Survival of G_2 individuals of FT and MD populations was 96% and 91%, respectively.

Laboratory rearing

Upon hatching, progeny were replaced individually in petri dishes 20 mm deep and 60 mm in diameter containing artificial lake water (Wyngaard and Chinnappa, 1982) and 10^4 cells/ml of *Cryptomonas ozolini* Sküjja (Starr Collection UTEX LB2194) isolated from Douglas Lake. The adult diet of *M. edax* was supplemented with the calanoid *Diaptomus dorsalis* (a common prey of *M. edax* in Lake Thonotosassa). *D. dorsalis* was mass cultured on *Chylamydomonas reinhardti* (Starr Collection No. 89) and artificial lake water. Algae and crustaceans were grown at 25 ± 0.5°C and a light:dark cycle of 14:10. Individual *M. edax* were transferred to clean dishes with fresh medium and food every other day, and individuals were observed daily for survival and development. Upon maturity, unrelated males were placed with females until the first clutch was produced. Animals were preserved in 4% formaldehyde for body size and clutch size measurements. Cephalothorax length was measured to the nearest 0.003 mm at 40× with an ocular micrometer.

Calculations of additive genetic variance components

The sire variance component estimates the phenotypic covariance of half sibs and thus estimates one fourth of the additive genetic variance, because half sibs have one fourth of their genes in common, on the average (Falconer, 1980). Estimates from half sibs provide the best unbiased estimate of additive genetic variance. Dam component estimates, which include variance due to dominance (non-additive), maternal effects, and common environment, in addition to the phenotypic covariance of half sibs, were calculated for comparison with the half sib estimates. Because individuals were reared individually after hatching, common family environment is not likely to be important. Upon reaching maturity, males and females were paired; the males were removed when the females produced eggs. Cannibalism of males by females after mating occurred occasionally in the FT matings and frequently in the MD matings, resulting in reduced numbers of males available for body size measurement. Sample size was too small to estimate the additive genetic variance of MD male body size. To minimize predation, some females and males were not mated but, instead, were preserved upon reaching maturity.

As is commonly the case in such studies, class sizes were unequal; this resulted from variation in sex ratio and (to a small degree) differential survival of offspring. All data were used, and variance components for additive genetic variances were computed using maximum likelihood estimation (MLE) procedure VARCOMP of the computer package SAS (Hemmerle and Hartley, 1973; Helwig and Council, 1979). This method of extracting parameter estimates differs from that used in ANOVA but assumes the same underlying genetic model. MLE is preferable to ANOVA, because unlike ANOVA, MLE does not result in negative variance components, which are unrealistic. The superiority of MLE to ANOVA for use on unbalanced data is discussed by Swallow and Monahan (1984). Standard errors of additive genetic variance components of half sibs were estimated according to Searle (1970). The significance levels of sire effects (or additive genetic variances) were tested by comparing the objective functions of the MLE analyses when the sire effects were estimated separately from the dam and within family variances to when they were combined with the dam variances. These objective functions closely approximate the mean squares (Hemmerele and Hartley, 1973), and their difference yields a Chi square value (χ^2) which can be used to determine significance levels using 2 d.f. I present and compare estimates of additive genetic variance (V_A) in preference to heritability estimates (h^2), which are a ratio of the additive genetic variance (V_A) to the phenotypic variance (V_P). It is the genetic variance that is of interest in the present study; comparisons of V_A are not complicated by the problems of comparing ratios. One trait, maturation time, was transformed to natural logarithms.

RESULTS

The half sib (sire) components of variance on which the heritability analyses were based will be discussed first, as they provide the least biased estimates of additive genetic variance, and full sib (dam) components of variance will be discussed later. Neither population showed any significant heritable variation of clutch size or male maturation time (Tables I, III). There was a significant heritable variation for female maturation time in the MD population, but not in the FT population (Table I). In

TABLE I

Estimates of maximum likelihood variance components of maturation time (days) of Mesocyclops edax *from Lake Thonotosassa, Florida (FT) and Douglas Lake, Michigan (MD). Maturation time was transformed to natural logarithms*

Population	Sex of offspring	Source of variation	*d.f.*	Variance component	(SE)	Percent of total variation	χ^2
FT	Female	Among sires	13	0.0	(0.001)	0.0	0
		Between dams, within sires	14	0.0050		39.1	
		Within families	204	0.0079		60.9	
		Total	231	0.0129		100.0	
MD	Female	Among sires	13	0.0163	(0.0077)	42.8	17.46***
		Between dams, within sires	14	0.0055		14.5	
		Within families	220	0.0162		42.7	
		Total	247	0.0381		100.0	
FT	Male	Among sires	13	0.0	(0.002)	0.0	0.01
		Between dams, within sires	14	0.0077		44.6	
		Within families	231	0.0096		55.4	
		Total	258	0.0173		100.0	
MD	Male	Among sires	10	0.0103	(0.010)	28.0	2.81
		Between dams, within sires	11	0.0178		48.3	
		Within families	117	0.0087		23.6	
		Total	138	0.0368		100.0	

*** $P < 0.001$.

contrast, there was significant heritable variation for female body size in the FT population but not in the MD population (Table II). The additive genetic variances of male body size could not be compared, because sample size was too small to obtain an estimate for the MD population (Table II).

The degree to which the variance component among full sibs exceeds that from the half sibs indicates, in theory, the contribution of maternal common environment and non-additive genetic dominance effects to the observed variation. Comparison of estimates based upon sire and dam components indicated considerable nonadditive genetic and/or maternal components for FT male and female maturation time MD male maturation rate (Table I) and clutch size (Table III). The only instance in which the sire and dam compounds approximated one another was for MD female body size. Three traits exhibited a dam variance considerably less than that of the sire variance: MD female maturation time and FT male and female body size.

DISCUSSION

The Florida (FT) population exhibited considerable additive genetic variance in body size, but not maturation time while the Michigan (MD) population showed the opposite trend. Both populations exhibited low additive genetic variance for clutch size. The standard errors of these variance components were large. However, in all cases (except maturation time of MD males) statistical tests of objective functions showed heritable variation only for traits that exhibited high amounts of additive genetic variance. Maturation time, body size, and clutch size influence survival and fecundity in natural populations and can be used to estimate the additive genetic variance of fitness components.

Before I discuss these genetic variance estimates, their limitations should be noted. Presumably these laboratory derived estimates of additive genetic variance reflect those

TABLE II

Estimates of maximum likelihood variance components of body size (μm) of Mesocyclops edax *from Lake Thonotosassa, Florida (FT) and Douglas Lake, Michigan (MD)*

Population	Sex of offspring	Source of variation	*d.f.*	Variance component	(SE)	Percent of total variation	χ^2
FT	Female	Among sires	14	31495	(4472)	13.7	9.09*
		Between dams, within sires	15	10409		4.5	
		Within families	160	187377		81.7	
		Total	189	229281		100.0	
MD	Female	Among sires	13	12210	(4472)	4.8	0.97
		Between dams, within sires	14	10906		4.8	
		Within families	174	231608		90.9	
		Total	201	254742		100.0	
FT	Male	Among sires	12	5849	(1348)	9.6	6.74*
		Between dams, within sires	13	0		0.0	
		Within families	125	54892		90.4	
		Total	150	60741		100.0	

* $P < .05$

TABLE III

Estimates of maximum likelihood variance components of clutch size of Mesocyclops edax *from Lake Thonotosassa, Florida (FT) and Douglas Lake, Michigan (MD).*

Population	Sex of offspring	Source of variation	*d.f.*	Variance component	(SE)	Percent of total variation	χ^2
FT	Female	Among sires	13	1.54	(3.75)	4.1	0
		Between dams, within sires	14	10.00		26.4	
		Within families	134	26.25		69.4	
		Total	161	37.78		100.0	
MD	Female	Among sires	10	0.00	(3.54)	0.0	0
		Between dams, within sires	11	5.58		24.3	
		Within families	74	17.44		75.7	
		Total	95	23.02		100.0	

of these populations in nature. The Florida population matures and reproduces over a wide temperature range (12–31°C) in nature, whereas the greater proportion of maturation and reproduction in the Michigan population occurs between 18–26°C. The lack of genotype-environment interactions, which would alter the additive genetic variances, is impossible to verify without repeating these experiments over a wide range of environmental conditions. Genotype-environment interactions have been reported for some life history traits in the marine copepod *Eurytemora herdmani* (McLaren, 1976) as well as for *Drosophila melanogaster* (Giesel *et al.*, 1980, but see Rose, 1984), but were absent for some traits in *D. pseudoobscura* (Tantawy, 1961). General remarks regarding the relevance of genotype-environment interaction to heritability studies are difficult to make, except that they can alter the interpretation of results and that they merit more investigation.

The observation of traits exhibiting high additive genetic variance in one population and low additive genetic variance in another is intriguing in itself. The mean values of these traits (maturation time, body size, clutch size) show genetically based differences between populations (Wyngaard, 1986). The two populations interbreed readily in the laboratory (Allan, 1984); thus these populations comprise one species and comparisons of their additive genetic variances are appropriate.

The trends in additive genetic variances reported in the present study may be meaningful in one or several regards. First, the amount of additive genetic variance of a trait may reflect the intensity of selection in the immediate past upon that trait. Fisher's Fundamental Theorem, which states that the rate of increase of mean population fitness at any time is proportional to the additive genetic variance in fitness (Fisher, 1958), provides a context in which the relative amounts of additive genetic variance estimated for traits in this study can be compared and discussed. Certainly the large standard errors around these estimates of additive genetic variance make comparisons between populations tenuous; however, the differences between populations in statistically detectable levels of additive genetic variance suggest such comparisons are worthwhile. The Michigan and Florida populations inhabit lakes that differ in temperature regime, food level, and predator abundance (Wyngaard *et al.*, 1982; Elmore, 1983; Wyngaard, 1986). The relative contributions of maturation time, body size, and clutch size to fitness in each population may be related to these differences between the lakes. Confounding selection pressures may have molded these life histories and contributed to obscuring any relationships that might exist among environmental variables and genetic variances. Nevertheless, a plausible and testable scenario can be conjectured that might explain, in part, the observed trends.

One possible explanation is that chronically low food levels in the Michigan lake places a premium on reaching a certain minimum body size, maximizing energy demands of growth and maturation. Small clutches may be the result of a tradeoff between clutch size and egg size, favoring a few large young and enhanced survival in a low food environment. In contrast, rapid maturation and large clutches may be favored in the Florida population, in which predation is intense and mortality falls heaviest upon juveniles (Charlesworth, 1980; Wyngaard *et al.,* 1982). The low additive genetic variances in maturation time and clutch size are consistent with this line of reasoning. At least, they suggest different potentials to respond to selection in the near future of microevolutionary time. Alternatively, low levels of additive genetic variance can result from random drift and founder effects. This latter explanation seems less likely, as plankton population sizes are typically large, *M. edax* is presumed to be highly vagile, and only small amounts of migration are necessary to overcome the effects of random drift.

High levels of additive genetic variance pose problems of interpretation because they can be attributed to a greater variety of causes: weak selection, no selection, changing selection, negative pleiotropy, to name a few. Others have compared heritabilities of various traits between populations in search of relationships between additive genetic variance and selection intensity. Berven and Gill (1983) argued that differences in heritabilities of larval duration and size at metamorphosis among three woodfrog populations suggested that selection intensities were very different in these populations. Arnold (1981) found similar heritabilities of feeding behavior in two geographically isolated snake populations.

The trends exhibited by maternal effects and/or non-additive genetic effects in this study are troublesome but not unusual. The lower dam relative to the sire components of MD female maturation time and FT female body size in the present study may reflect non-random mating among parents with respect to genotype. If sires were mated with dams of similar genotype for a quantitative trait, then the genetic variance contributed by the dam would be masked in a nested design such as that used in this study. The results for *M. edax* probably represent a conservative estimate of the extent to which dominance and maternal effects are present in these traits.

Perhaps the most important issue is whether the level of additive genetic variance reflects the potential response of the population to selection. The moderate and high heritabilities of female and male, respectively, temperature tolerance in the estuarine copepod *Eurytemora affinis* provided an opportunity to determine whether such heritable variation was available to be acted upon by selection. Ketzner and Bradley (1982) exposed *E. affinis* to three different constant temperature regimes and to three variable temperature regimes over a two year period (20–70 generations) and demonstrated genetic divergence among the regimes as well as a decline in the additive genetic variance in one of the selected lines (Bradley and Ketzner, 1982). Other studies using selection experiments to address this question include Istock *et al.* (1975), Dingle *et al.* (1977), Doyle (1978), and Doyle and Hunte (1981). This growing body of empirical studies (review in Dingle and Hegmann, 1982) reveals considerable additive genetic variance to be present in natural populations of many species. The present study is consistent with these reports in that some of the life history traits examined here exhibited considerable additive genetic variance. The suggested contrasting patterns of additive genetic variance present in particular traits between the two *M. edax* populations indicate the need for additional studies of heritable variation of life history traits in natural populations. Explanations of additive genetic variance are complex and will likely require both examination of genetic correlations and very carefully designed selection experiments as discussed by Istock (1982), Lande (1982), Rose (1982) and Lande and Arnold (1983).

ACKNOWLEDGMENTS

I am grateful to K. Berven, B. Bradley, J. Coyne, C. Istock, D. Reznick, M. Rose, and W. Walton, and for stimulating discussion and helpful comments on this manuscript. J. D. Allan provided inspiration, encouragement, support, and cooperation throughout the entire study. R. Stemberger provided the algae used to culture the copepods and much appreciated expertise in algal culture. I thank J. Beechum and T. McTigue for their careful assistance in the laboratory, L. Douglass, B. Mock, E. Russek, and J. Travis for statistical and computer advice, and B. Cowell and C. Hull for collecting and shipping plankton. This work was supported by NSF-DEB80-04450 and University of Maryland Computer Services grant to J. D. Allan and a Sigma Xi Grant-in-Aid for Research to the author.

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BEHAVIORAL, ELECTROPHYSIOLOGICAL, AND MORPHOLOGICAL INVESTIGATIONS OF STATOCYST FUNCTION IN THE NUDIBRANCH MOLLUSC *HERMISSENDA CRASSICORNIS*

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ABSTRACT

Laboratory-reared specimens of the nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) possess statocysts that contain either one, several, or a species-typical number of statoconia (150–200). The number of statoconia per statocyst is significantly related to the growth rate of the animal during its first month post-metamorphosis, with slower-growing individuals (<0.88 mm/day) tending to possess a single statoconium bilaterally. Laboratory-reared animals with statocysts containing a single statoconium exhibited significantly weaker conditioning effects as expressed by faster response latencies to enter an illuminated area one and two days following behavioral training as compared with laboratory-reared animals with more than one statoconium per statocyst. Rotation of the isolated nervous systems of preparations with a single statoconium revealed that caudal hair cells exhibited reduced depolarization and the hyperpolarization of Type B photoreceptors in response to rotation was absent. As the number of statoconia increased, caudal hair cell depolarization and type B cell hyperpolarization occurred with increasing frequency. Cell responses to rotation were identical to those recorded from wild-type cells when the number of statoconia exceeded eight.

Statocysts from laboratory-reared animals with a single statoconium possess the same number (13) of sensory (hair) cells, but differ from typical statocysts (150–200 statoconia) in that they are about half the normal size, the lumenal diameter is reduced, the hair cells are about twice as thick, and the sensory cilia are closer together. Both ciliary morphology and basic histology are similar in laboratory-reared and field-collected animals.

Decreased mechanotransduction is postulated to be primarily responsible for the changes in behavioral conditioning and sensory cell activity observed in these laboratory-reared animals. This decreased sensory transduction results from reduction in statoconia number and restricted movement of the stones in statocysts with reduced lumina.

INTRODUCTION

The paired statocysts of the Pacific Coast nudibranch mollusc *Hermissenda crassicornis* (Eschscholtz, 1831) are spherical fluid-filled structures containing a mass of

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Received 27 June 1985; accepted 3 January 1986.

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discrete stones (statoconia), which interact with the tips of cilia projecting from the sensory (hair) cells lining the lumen (Alkon, 1975; Grossman *et al.*, 1979; Kuzirian *et al.*, 1981). Statocysts of this type, common in gastropod molluscs, are generally considered to be mechanoreceptors (Wolff, 1975). Intracellular recordings from hair cells during statocyst displacement or during rotation of the isolated nervous system, have shown *Hermissenda* statocysts to be sensitive to an increase in gravitational force (Alkon and Bak, 1973; Alkon, 1975; Tabata and Alkon, 1982; Goh and Alkon, 1984). Electrophysiological analysis of the functional inter-relationships between the visual and mechanoreceptive pathways has been facilitated by the relatively small number of neurons comprising these two sensory networks (Alkon, 1974a; see review by Alkon, 1980). These two sensory systems are important behaviorally since a conditioning procedure consisting of illumination paired with rotation results in a long-term modification of phototactic behavior in *Hermissenda* (Alkon, 1974b; Crow and Alkon, 1978; Farley and Alkon, 1982; Crow and Offenbach, 1983; Crow, 1983, 1985; Harrigan and Alkon, 1985).

Hermissenda was reared in the laboratory under defined environmental conditions (Harrigan and Alkon, 1978) to provide animals of known history for similar behavioral and neurophysiological studies. Crow and Harrigan (1979) found that laboratory-reared animals showed significantly less behavioral variability in response to light before training, and in some cases following the training procedure. Dissection of these laboratory-reared animals revealed variation within and between individuals in the number of statoconia per statocyst, ranging from one to the typical complement of 150–200 per statocyst. Deformed or absent statoliths in hatchlings of eight cephalopod species that underwent embryonic development in artificial seawater in the laboratory have been reported (Colmers *et al.*, 1984), and veliger larvae of the opisthobranch mollusc *Aplysia californica* have been shown to require the addition of the element strontium to artificial seawater for normal shell and statolith development prior to hatching (Bidwell *et al.*, 1986). In our study, *Hermissenda* larvae cultured in natural sea water possessed normal shells and one statoconium per statocyst; morphological variations in statocysts were observed only in post-metamorphic animals and were correlated with antibiotic treatment (Crow and Harrigan, 1979).

Alkon (1975), Grossman *et al.* (1979), Grossman and Alkon (1983), and Stommel *et al.* (1980), in separate investigations of sensory transduction in the statocyst, concluded that hair cell voltage noise and generator potentials arise in response to the force exerted on the hair cell sensory cilia by the weight of the mass of statoconia. In those studies, occasionally one of the paired statocysts from a field-collected *Hermissenda* was found to contain a single statoconium. The baseline hair cell noise was reduced in these animals (Grossman *et al.*, 1979).

Crow and Harrigan (1979) suggested that because animals with a single statoconium appeared to be less affected by training with paired stimulation, the study of laboratory-reared animals with variable numbers of statoconia would aid in clarifying the role of the statocyst in mediating the behavioral change produced by conditioning. In the present study we will first describe the relationship between laboratory culture conditions, growth rates of the animals, as well as the morphology of the adult statocyst, including number of statoconia. Next, we will present behavioral, morphological, and neurophysiological evidence from these same animals which demonstrates a relationship between the presence of a single statoconium, diminished effectiveness of the conditioning procedures, and reduction in the size of the hair cell generator potential. Changes in photoreceptor responses to rotation in the case of the single statoconium are also described.

MATERIALS AND METHODS

Culture conditions

Wild *Hermissenda* were provided by M. Morris, Sea Life Supply, Sand City, California. Animals were maintained in individual containers in a flow-through seawater system at 13°–15°C. Initially, egg masses were obtained from parents within one week of arrival in the laboratory and incubated until hatching at 14° ± 2°C under a 12 hour light/dark cycle in 200 ml of 0.22 μm-filtered seawater, changed daily.

Adults were isolated upon arrival in the laboratory and the first egg masses deposited by each were discarded to determine the condition of the parents' statocysts relative to those of the offspring. Previous observations (Harrigan) showed that animals isolated on arrival in the laboratory, usually 3–5 days after collection, tended to deposit one large fertile egg mass within 4 days after isolation. Subsequent egg masses laid by isolates had a low percent fertilization and were often non-viable. Rutowski (1983) found that individual *Hermissenda* isolated after copulation store enough sperm to fertilize 2–3 egg masses; physiological stresses associated with collection and shipment may have reduced gamete viability or stimulated production of especially large egg masses after isolation in our laboratory, resulting in relative sperm depletion after the first egg mass was deposited. After egg-laying, parents were placed in groups of four to five in a refrigerated aquarium (Dayno Mfg. Co., Lynn, Massachusetts) at 13°–15°C and fed tunicate viscera (*Ciona intestinalis*). Subsequent egg masses were assumed to have been fertilized predominantly by members of the group, all of which were then dissected and the statocyst condition recorded. This procedure was repeated three times so that about half the animals used in the present study were reared from egg masses deposited by parents of known statocyst type.

Animals were cultured in the laboratory through the planktonic veliger stage to the benthic juvenile and adult stages as previously described by Harrigan and Alkon (1978). All cultures were started from a mixture of veligers from at least 2–4 egg masses. Twenty percent of the individuals included in the present study were cultured in 5 μg/ml of the antibiotic chloramphenical (Sigma); the remainder were exposed to 5 μg/ml of rifampicin (Sigma). Unless otherwise noted, all animals were cultured under a 12 hour light/dark cycle at a light intensity of $3–5 \times 10^3$ ergs cm^{-2} s^{-1} and at 13°–15°C.

To determine if light intensity and photoperiod affected statocyst development, three egg masses deposited by wild specimens of *Hermissenda* were divided in half; each half was maintained from the two-cell stage through hatching in either constant light of 8×10^3 ergs cm^{-2} s^{-1} or 15 minutes light:23.75 hours dark. Larval and post-metamorphic stages were cultured in the same illumination condition experienced prior to hatching, and in 5 μg/ml rifampicin.

All post-metamorphic animals were maintained individually in 100 ml of standing seawater, with or without antibiotic, and changed three times per week initially and then daily when the animals reached 1 cm body length. Animals were fed an excess of tunicate viscera daily, so that growth rates were not food limited. At 30–50 days post-metamorphosis, adults were transferred to a slow flow-through seawater system (1 liter/min, 15°C), and kept individually on a light cycle of 6.5 hours light (at 6×10^3 ergs cm^{-2} s^{-1}):17.5 hours dark during behavioral training and testing.

Body length was measured from the anterior end of the animal, at a point halfway between the oral tentacles, to the tip of the tail when the animals were fully extended and moving forward (Risso-Dominguez, 1963). Photomicrographs and measurements of very small animals and statocysts were made through a Zeiss Universal microscope with a calibrated ocular micrometer. Growth rates were compared using either Student's t-test (t) or analysis of variance (F) for unequal sample sizes (Snedecor and Cochran, 1967).

Electron microscopy

Animals were dissected and whole brain preparations were prepared according to previously described methods (Alkon, 1975). After the preparations were tested electrophysiologically the incubation medium was poured off and two to three drops of either of the following fixatives were placed onto the pinned brain preparations: 3% glutaraldehyde in either 0.1 M sodium cacodylate buffer (pH 7.3); 0.1 M sodium phosphate buffer plus 5 mM $MgCl_2$ and 5 mM EGTA (pH 7.4); 0.1 M HEPES buffer plus 5 mM $MgCl_2$ and 5 mM EGTA (pH 7.4). In all cases the osmotic pressure was adjusted to 1100 to 1200 milliosmoles with sucrose. After allowing the fixative to remain on the preparations for two to three minutes, the pins were removed and the entire brains placed in vials containing fresh solutions of their respective fixatives (30 min, 20°C). Following fixation the tissues were rinsed in their respective buffer compositions minus glutaraldehyde. In the final wash, the brains were bisected and all extraneous connectives removed. Post-fixation was carried out for 30 to 45 minutes (20°C) in a buffered 2% osmium tetroxide solution followed by straight buffer and distilled water washes before dehydrating in a graded ethanol series. Propylene oxide was used as a transitional solvent prior to infiltrating and embedding the tissue in an epon-araldite mixture (Geiselman and Burke, 1973). Hardened plastic blocks were subsequently sectioned with a diamond knife (Dupont Corp.) on an LKB 8800 Ultrotome III. Thick sections (1.0–2.0 μm) used for orientation and sensory cell counts were mounted on glass coverslips and stained with methylene blue or toluidine blue in 1% sodium borate (70°C). This sections were stained with aqueous uranyl acetate and lead citrate or vanadatomolybdate (Callahan and Hornes, 1964), and viewed on a Philips EM 300 or EM 400T transmission electron microscope. Live whole-mount brain preparations and sectioned material were observed and photographed on a Zeiss Universal microscope equipped with differential interference contrast optics.

Behavioral training and testing

The behavioral training apparatus and testing procedures for photopositive behavior have been described previously (Crow and Alkon, 1978; Tyndale and Crow, 1979; Crow, 1983, 1985; Harrigan and Alkon, 1985). Briefly, the time (latency) taken by laboratory-reared animals to move the length of 228 mm long seawater-filled glass tubes from very dim to brighter illumination (white light, 5.2×10^3 ergs cm^{-2} s^{-1}) was recorded automatically by photocells located beneath the glass tubes in the center of the turntable and connected to an event recorder. The glass tubes were attached to clips on a modified turntable so that one end, the start, was at the periphery (dim light) and the other end, the brightly illuminated area, faced the center of rotation. After two days of baseline testing to determine the latencies to move into the illuminated area the animals were placed on an automated training schedule consisting of 50 trials of diffuse light (30 s) paired with rotation (30 s at 2.24 g) of the turntable. After three consecutive days of training (total trials = 150) the animals' response latencies to enter the illuminated area were remeasured at the conclusion of the last training session and again at 24 and 48 hours.

It was previously shown for field-collected animals (Crow and Alkon, 1978; Farley and Alkon, 1982; Crow and Offenbach, 1983; Crow, 1983, 1985; Harrigan and Alkon,

1985) and for laboratory-reared animals (Crow and Harrigan, 1979) that response latencies of trained animals were significantly longer following training as compared with baseline tests and with various control groups used to assess non-associative contributions to the change in phototactic behavior.

Intracellular recordings

Recordings using standard intracellular techniques were made simultaneously from the type B photoreceptors and hair cells of isolated nervous system preparations from eleven of the laboratory-reared animals containing statocysts with either one, several (4–7), or a typical number of statoconia (150–200). Details of the dissection, isolation of the nervous systems, and recording methods have been reported elsewhere (Alkon, 1973, 1975). Rotation of the isolated nervous system while recording intracellularly from statocyst hair cells followed established experimental procedures (Alkon, 1975). Recordings were made about 24 hours after the last training session, and reflected the long-term associative effects of behavioral training.

RESULTS

Effect of culture conditions on statocyst development

Statocysts form during intracapsular development, in the late trochophore and early veliger stages. Larval statocysts, which in newly hatched veligers measure 16.3 ± 1.1 μm diameter ($n = 20$) uniformly contain a single statoconium measuring 9.2 ± 0.7 μm diameter ($n = 20$). In 45% of the laboratory-reared animals included in the present study, a single statoconium was retained in the adult stage in both statocysts.

Antibiotics, either 5 μg/ml chloramphenicol or 5 μg/ml rifampicin, were routinely added to the culture water to increase percent survival of larvae and juveniles. Growth rate in the first month post-metamorphosis, when individuals (isolated) were maintained in standing seawater culture containing either antibiotic, was an important factor in predicting the number of statoconia in the adult statocyst, even though most adults were dissected at 50–90 days post-metamorphosis after two or more weeks in flowing seawater without antibiotic.

Growth is slow for the first two weeks post-metamorphosis, averaging 0.08 mm/day ($n = 10$), perhaps reflecting energy requirements for the major changes in body structure accompanying metamorphosis. Subsequent increase in body length is approximately constant, averaging 0.70 mm/day ($n = 10$), until about 90 days post-metamorphosis when some individuals become senescent. At one month post-metamorphosis mean individual body length was 2.45 cm (range = 0.48–4.45 cm, $n = 10$). Dissection of ten small wild individuals between 1.00 and 1.50 cm long, which by extrapolation from growth rates of laboratory-reared animals would be within one month post-metamorphosis, revealed only typical adult statocysts with no apparent reduction in number of statoconia. This observation suggests that in wild populations statoconia formation is maximal in the first month post-metamorphosis.

Although the parents of half the laboratory-reared population of *Hermissenda* were dissected and found to contain typical statocysts, there was considerable variation in number of statoconia among their first-generation offspring. Growth rates (in mm/day) were compared, in the presence or absence of antibiotic, for animals with a single statoconium, several statoconia (including any case in which different numbers of stones were found in the paired statocysts), or with the typical number of statoconia per statocyst (Table I). Twelve of the 32 individuals with a single statoconium in the 'antibiotic' treatment were exposed to chloramphenicol and the rest to rifampicin.

TABLE I

[illegible] *in first month post-metamorphosis*

	No Antibiotic			Antibiotic		
	n	mm/day	Percent of total	n	mm/day	Percent of total
1. Single stone	3	0.63 ± 0.09	27	32	0.65 ± 0.27	48
2. Several stones	3	0.63 ± 0.28	27	23	0.81 ± 0.26	35
3. Typical	5	0.85 ± 0.08	46	11	0.97 ± 0.31	17
Total:	11			66		

Because there was no significant difference between their growth rates ($t_{30} = 0.631$, $P > 0.05$) the two samples were combined.

Table I illustrates the following points. First, in both the 'antibiotic' and 'no antibiotic' treatments animals with slower growth rates (<0.80 mm/day) retain the single statoconium condition as adults, whereas faster-growing animals (>0.80 mm/day) tend to have a typical complement of statoconia. Second, mean growth rates of antibiotic-treated animals, when separated into categories consisting of those with one, several, or a typical number of statoconia per statocyst, were overall significantly different ($F_{2,73} = 6.30$, $P < 0.01$). The Newman-Keuls test for ordered means showed a significant difference ($P < 0.05$) only between the mean growth rates for animals with a single statoconium and animals with typical statocysts. Mean growth rates for animals in the 'several' category were not significantly different from those of animals in the other two categories. Growth rates of animals with a single statoconium in the 'no antibiotic' treatment were significantly different from those of animals with typical statocysts ($t' = 3.50$, df = 4, $P < 0.05$). Third, a higher proportion (46%) of animals in the small 'no antibiotic' treatment grew relatively faster and had typical statocysts than in the 'antibiotic' treatment (17% with typical statocysts).

These data support the conclusion that exposure to either chloramphenicol or rifampicin at low concentrations (5 μg/ml) retards the growth rate to a variable extent in different individuals. There is also a significant positive relationship between the slowest growth rates and the retention of a single statoconium in the adult stage.

When animals from parents with typical statocysts, cultured from fertilization to maturity either in constant light of 8×10^3 ergs cm^{-2} s^{-1} (n = 7) or 15 minutes of room light per day (n = 4), were dissected, all were found to possess a single statoconium. Growth rates in the first month post-metamorphosis were relatively slow in each illumination condition, and corresponded to rates associated with the single statoconium condition in animals reared in 12 hours light/dark (LL = 0.40 ± 0.18 mm/day; 15 min L = 0.59 ± 0.23 mm/day).

Statocyst morphology

Animals with reduced numbers of statoconia or a single statoconium showed the same general statocyst morphology as animals with the standard complement. Cell counts done on stained thick sections (1–2 μm) revealed the usual number of 13 sensory (hair) cells. Interspersed between the sensory cells are the non-sensory support cells. The sensory kinocilia (hairs) had the same typical length (8–10 μm) and breadth (0.3 μm) found in statocysts containing the typical number of statoconia (150–200). Exact numbers of cilia/cell were not determined but the average spacing between cilia was 1.4 μg. The ciliary morphology appeared within normal limits of that seen in the field-collected animals, including the basal bodies with their elaborate rootlet system located just below the apical surface of the sensory cell (Kuzirian *et al.*, 1981). The basic histology of the cytoplasm and organelles of both the sensory and support cells was also unremarkable (see Figs. 1–4).

The principal pathology demonstrated in statocysts from animals ($n = 6$) reared under laboratory conditions was dimensional. Statocysts containing reduced numbers of statoconia or a single statoconium were smaller. They averaged 40 to 55 μm in overall external diameter (90%: 45–50 μm, fixed preparations) which is approximately half the normal size. Concomitant with the overall size reduction, the lumenal diameter was reduced by four to five times, depending upon the number of statoconia present (10–25 μm, fixed preparations). The sensory cells, rather than being typically flattened (5–10 μm thick) with large, elongate nuclei, were 2.0 to 2.5 times thicker (13–17 μm) and possessed large oval to round nuclei. This increase in thickness was due solely to an increase in the amount of granuloplasm present within each sensory cell (*cf.* Geuze, 1968; Stahlschmidt and Wolff, 1972). Spacing between the sensory cilia (1.4 μm) was only half the distance found in typically developed statocysts. Observations made on live dissected preparations using differential interference contrast (Nomarski) optics indicated a reduced and/or restricted movement of the statoconia due to the small size of the lumen.

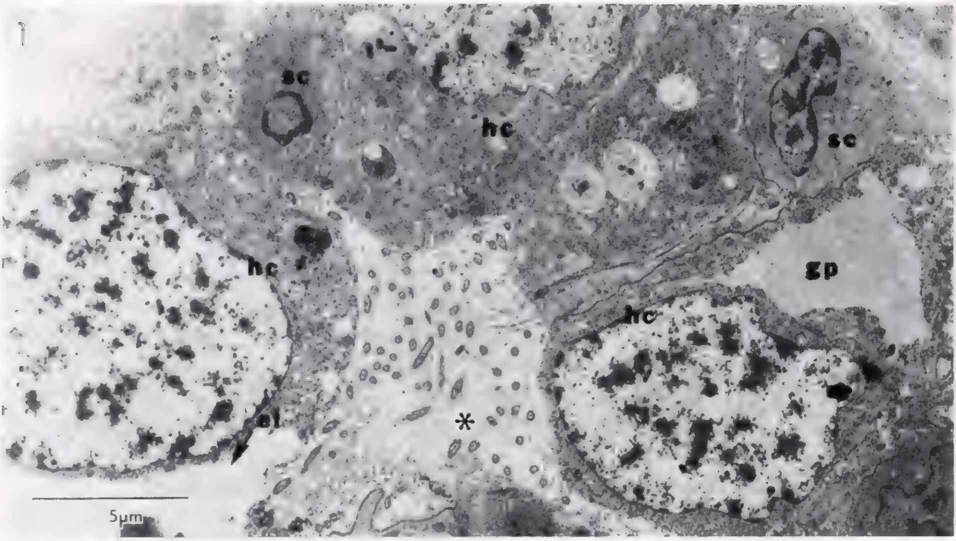
The reduced movement of the stones could also be due to the presence of an intraluminal matrix which was found in some specimens (Fig. 5). This organic matrix completely surrounded the stones. Additionally, the dense protein matrix layers normally composing the stones found in field-collected animals (Fig. 5, insert) is greatly reduced, much less dense and very loosely organized, or in some stones almost entirely absent. The interluminal protein matrix coupled with the lack of organization of the intra-statolith protein matrix may be indicators of a major perturbation of normal protein chemistry brought about by laboratory culture conditions and the principal suspected cause of the observed pathology.

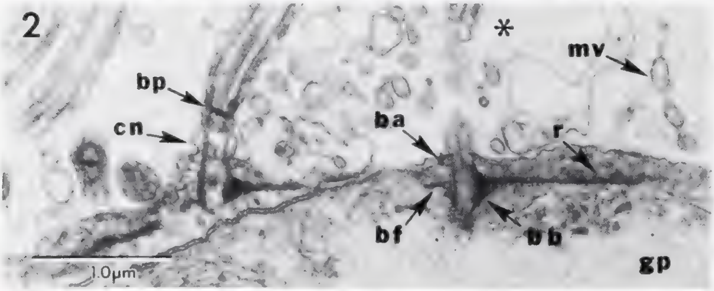
Behavior

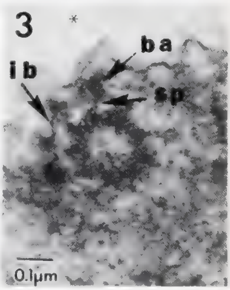
Following measurement of phototactic behavior after behavioral training (see Materials and Methods), the laboratory-reared specimens of *Hermissenda* were dissected and the two statocysts examined. Animals were divided into groups, those with several or a typical number of statoconia and animals with one statoconium. Dissections were done blind so that the experimenter did not know the results of the behavioral tests for the individual animals.

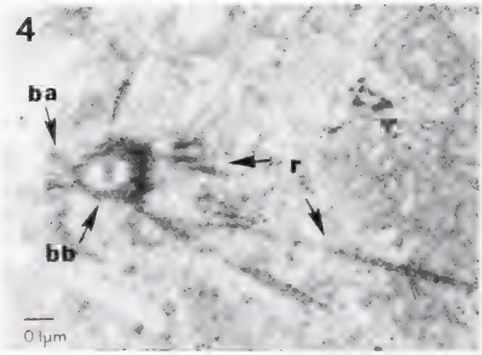
There were no significant differences in behavior before or after training for animals from the 'no antibiotic' and 'antibiotic' groups. However, in contrast to the 'antibiotic' treatment, there was a significant difference in the latencies to enter light between animals with a single statoconium and those with several to typical numbers of statoconia (Mann-Whitney U-test, $n = 19$, $P < 0.02$; Hollander and Wolfe, 1973). The response latencies before training were not different for animals with variable numbers of statoconia.

Laboratory-reared specimens of *Hermissenda* with a single statoconium exhibited faster response latencies following training as compared to animals with a typical or reduced complement of statoconia (Fig. 6). The reduced effectiveness of the training procedure for *Hermissenda* specimens containing a single statoconium is consistent with observations made on field-collected animals that had statocysts containing a single statoconium (Crow and Harrigan, 1979).









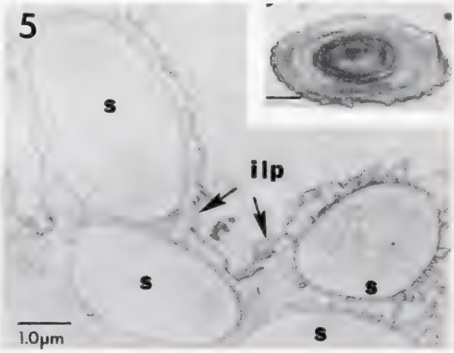


FIGURE 1. Low-power electron micrograph of statocyst from a laboratory-reared animal illustrating the anomalous morphology of the (1) thickened sensory hair cells (hc) with their large round nuclei and in the cell at the right, a portion of the increased amount of granuloplasm (gp) and (2) the reduced size of lumen (*) into which the sensory cilia project: el, electrode lesion, indicating penetration site of microelectrode used for electrophysiologic testing (basement membrane removed by protease digestion to facilitate microelectrode penetration).

FIGURE 2. Longitudinal section of two kinocilia from two adjacent hair cells demonstrating the normally expected appearance: ba, button anchor; bb, basal body; bf, basal foot; bp, double basal plate indicating termination point of central axoneme; cn, ciliary necklace region; gp, granuloplasm of hair cell; *, statocyst lumen; mv, microvillus, projecting from hair cell's apical surface; r, laterally projecting rootlet system.

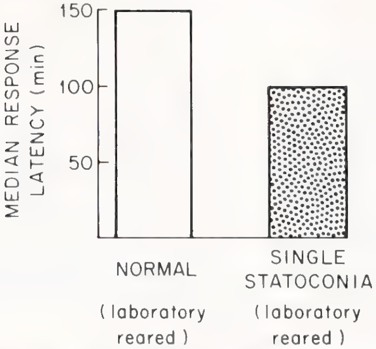


FIGURE 6. Median response latencies of laboratory-reared *Hermissenda* individuals with a normal complement of statoconia compared to those with single statoconia tested immediately following three days of training.

These results from laboratory-reared animals with a single statoconium may help to elucidate the involvement of the statocysts in the modification of phototactic behavior since the contribution of non-specific sensory input resulting from rotation would not be expected to change in these preparations.

Electrophysiology

Statocyst hair cells and photoreceptor cells synapse directly onto each other and also converge onto interneurons in the optic and cerebropleural ganglia (Tabata and Alkon, 1982; Alkon, 1983; Fig. 7). Intracellular recordings from caudal hair cells and/or ipsilateral Type B photoreceptors were obtained from the isolated circumesophageal nervous systems of *Hermissenda* (Alkon, 1975). The isolated nervous systems were oriented with respect to the center of rotation such that rotation caused the statoconia, as they experience angular acceleration, to exert pressure on the hairs of the caudal cells in the two statocysts. For statocysts with a typical number (150–200) of statoconia, rotation results in a depolarizing generator potential and an increase in spike frequency recorded from the cell bodies of caudal hair cells as previously reported (Alkon, 1975). When the response to rotation is recorded from a simultaneous penetration of a caudal hair cell and an ipsilateral Type B photoreceptor, the response recorded from both cells is a graded function of the number of statoconia (Fig. 8A, B, C). In statocysts with only a single statoconium the depolarization in response to rotation ($\leq 0.9\ g$) was greatly reduced or did not occur (Fig. 8C; 9A; Table II). As

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FIGURE 3. Tangential section just below hair cell's apical surface illustrating four of the nine button anchors (ba) with their spoke connections (sp) to the triplet tubules of the basal body as well as the osmiophillic interconnecting bridges (ib) between them (*, statocyst lumen).

FIGURE 4. Slightly oblique section through the hair cell's apical surface demonstrating the asymmetrical elaboration of the basal body (bb) rootlet system (r) in a form resembling an astral array; ba, button anchor with spoke connection to basal body (*, statocyst lumen).

FIGURE 5. Micrograph of several cross-sectioned statoconia (s) from a statocyst containing a greatly reduced number of stones illustrating intraluminal protein matrix (ilp) binding stones together and demonstrating the lack of density and organization of the protein matrix typically seen in statoconia of field-collected animals (insert: scale bar = 1.0 μm).

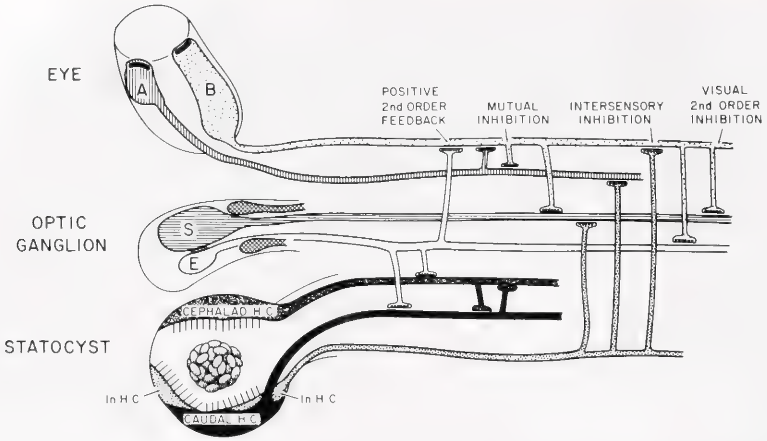

FIGURE 7. Schematic diagram of connections between the visual and statocyst pathways, as determined from intracellular recordings. Note that sensory cell interactions are primarily mutually inhibitory. Caudal hair cells synaptically inhibit the type B photoreceptor; type B cell inhibits second-order visual cells in the optic ganglion, resulting in excitation of the cephalad hair cell and inhibition of the caudal hair cell. A, B: type A and type B photoreceptor cells. S, E: optic ganglion cells. H.C.: hair cell (from Alkon, 1979).

statoconia number increased above three, the amplitude of the depolarizing response evoked by rotation was increased (Fig. 8B).

With the same orientation of the nervous systems as just described (the caudal orientation) Type B photoreceptors in preparations with typical statoconia number usually hyperpolarize in response to rotation (Fig. 8A; 9B; also, Tabata and Alkon, 1982). For Type B cells from preparations having statocysts with only a single statoconium, this hyperpolarizing response to rotation (≤ 0.9 *g*) was absent (Fig. 8C, upper trace; Table II). As statoconia number increased above three, the hyperpolarizing response of the ipsilateral Type B photoreceptors was evident although not of the same magnitude as the response from typical statocysts (see Fig. 8A, B). Simultaneous recordings from type B cells and caudal hair cells containing eight or more statoconia could not be distinguished from those obtained from field-collected animals, indicating that hair cell-photoreceptor synapses function normally in laboratory-reared animals with several or many statoconia. The degree of synaptic inhibition between caudal hair cells and ipsilateral type B cells was similar in all preparations and independent of statoconium number.

Tabata and Alkon (1982) recorded from 14 caudal hair cell-Type B photoreceptor pairs from animals with typical statocysts; in these cases B-cell inhibition occurred in 8 out of 14 pairs. Synaptic inhibition of Type B cells by caudal hair cells can account, therefore, for the hyperpolarizing response of Type B cells to rotation (*i.e.*, in the caudal orientation; Fig. 9B). However, a change in this typical synaptic interaction cannot account for the absence of Type B hyperpolarizing responses observed from preparations with single statoconium statocysts (Fig. 8C; 9A). With single statoconia, synaptic inhibition of Type B cells by caudal (ipsilateral) hair cells was readily demonstrated (Fig. 9C; Table II, 3 out of 4 preparations). This suggests that the absence of a hyperpolarizing response in the B photoreceptor as evoked by rotation is due to the ineffectiveness of the *g* forces used to produce a depolarizing generator potential in studies of these nervous systems.

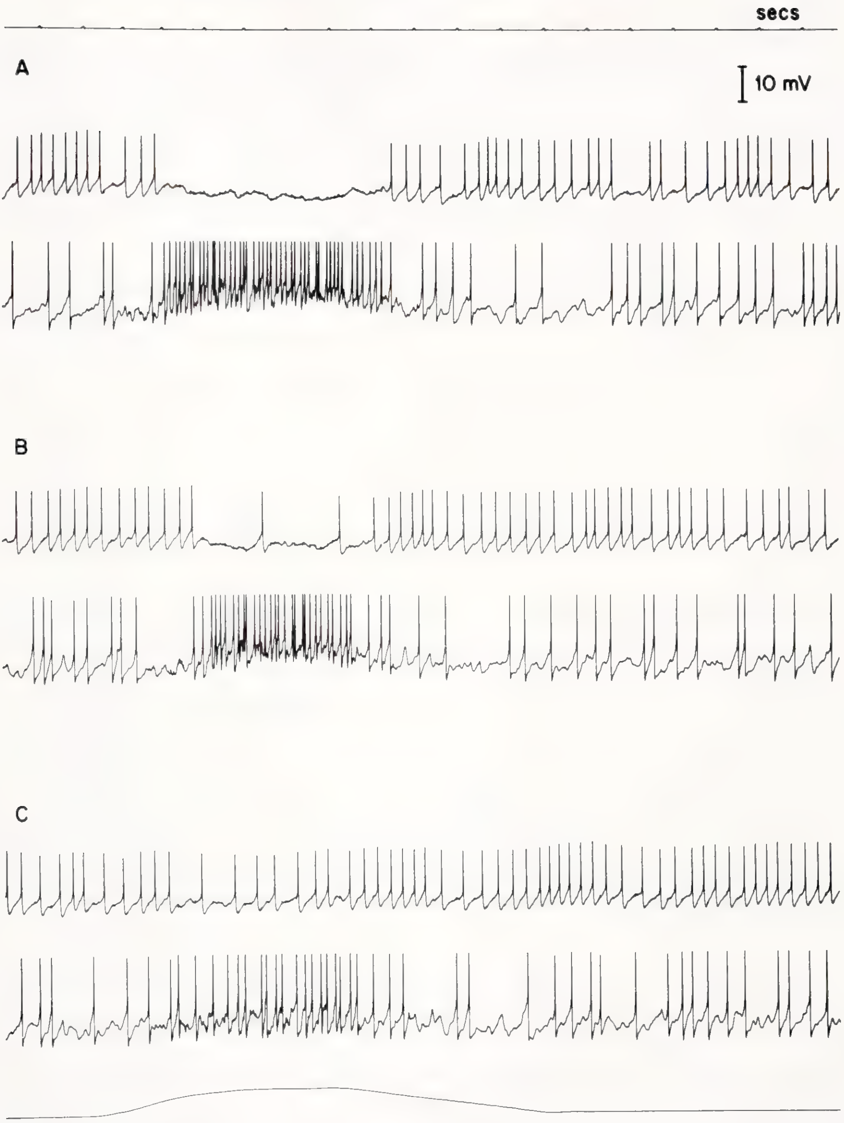

FIGURE 8. Intracellular recordings from type B ipsilateral photoreceptors (upper traces) and caudal hair cells (lower traces). Single trace at the bottom illustrates the time course of the rotational stimulus. A. Preparation containing typical complement (150–200) statoconia. B. Preparation with several statoconia. C. Preparation containing a single statoconium.

Together, these results clearly indicate that for single statoconium preparations, Type B cells do not hyperpolarize in response to rotation (≤ 0.9 *g*) because ipsilateral caudal hair cells do not show their normal depolarizing responses. Presumably with somewhat higher *g* force such as that used in training (2.24 *g*) caudal hair cells from statocysts with a single statoconium would show some depolarizing response, albeit of much smaller than normal magnitudes.

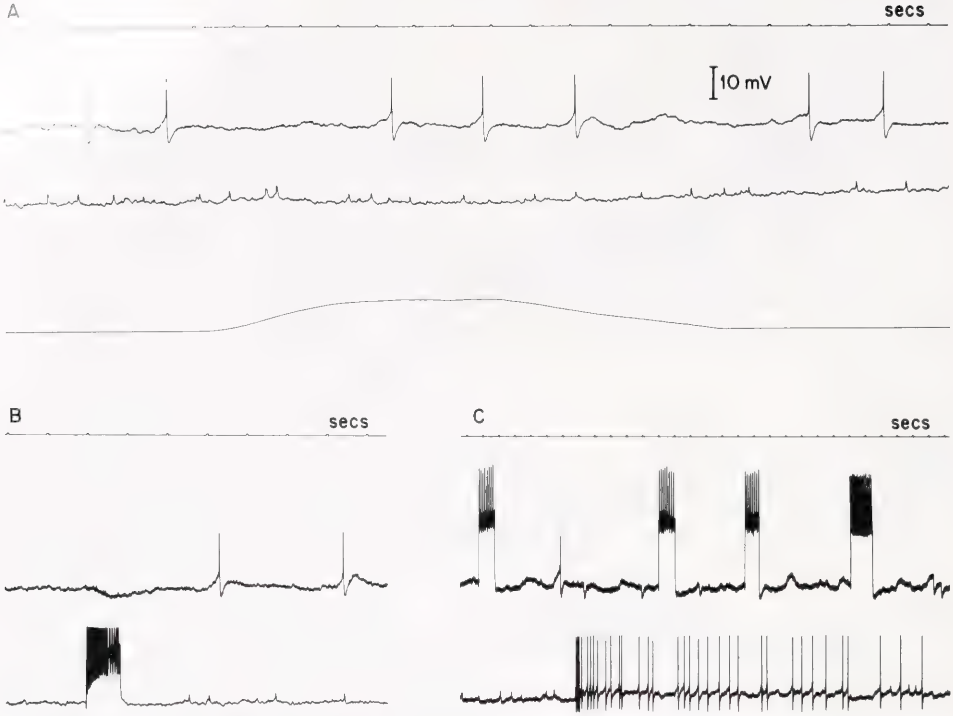

FIGURE 9. A. Intracellular recording from Type B photoreceptor (upper trace) and caudal hair cell (lower trace) in a preparation containing a single statoconium. Single trace at the bottom illustrates the time course of the rotational stimulus demonstrating the absence of a generator potential in response to rotation ($g = 0.9$). B. Synaptic inhibition and hyperpolarization of Type B photoreceptors resulting from an electrically produced firing burst in a caudal hair cell preparation with typical number of statoconia. C. Synaptic inhibition of a Type B photoreceptor resulting from electrical stimulation of an ipsilateral caudal hair cell in a statocyst containing a single statoconium.

DISCUSSION

Although antibiotics are used routinely to control bacterial populations in marine invertebrate culture systems (Smith and Chanley, 1972), little is known about their physiological effect on growth and morphology of the developing animal. Biochemical investigations have shown that both chloramphenicol and rifampicin inhibit protein synthesis in eukaryotes (Corcoran and Hahn, 1975). These antibiotics, used in the present study at 5 μg/ml each, inhibited the post-metamorphic growth rate of *Hermissenda* relative to growth rates in the absence of antibiotic. Both antibiotics were applied within the reported concentration range for effects on eukaryotic systems (Corcoran and Hahn, 1975).

Antibiotic-induced inhibition of growth rate was the only variable that was significantly related to the number of statoconia per statocyst. With few exceptions, the slowest-growing animals possessed statocysts containing a single statoconium. Because three out of eleven animals maintained in the absence of antibiotics contained a single statoconium in each statocyst, and because a similar sample of very small (1.0–1.5

TABLE II

Responsiveness of Type B photoreceptors and caudal hair cells to rotation (0.4, 0.5, 0.6 g) or to reciprocal stimulation in preparations with 1, several, or > 8 statoconia

0 = No response 1 = Response ± = Threshold response

	Type B hyperpolarization				Caudal hair cell depolarization					
	0.4	0.5	0.6	g	0.4	0.5	0.6	g	H.C. → B.	B. → H.C.
Single statoconium										
1.	0	0	0		0	0	0		0	0
2.	0	0	0		0	±	±		1	1
3.	0	0	±		0	±	±		1	1
4.	—	—	—		0	0	0		—	—
5.	0	0	0		0	0	0		1	1
3–8 statoconia										
6.	0	±	±		0	0	±		1	0
7.	1	1	1		1	1	1		1	1
8.	0	0	0		1	1	1		0	—
9.	0	0	0		—	—	—		0	0
10.	0	0	0		1	1	1		—	—
>8 statoconia										
11.	1	1	1		—	—	—		0	1
12.	1	1	1		1	1	1		1	0
13.	—	—	—		1	1	1		—	—

H.C. = Hair cell.
B = type B photoreceptor.

cm) wild *Hermissenda* uniformly contained typical statocysts, other variables such as maintenance in standing *versus* flow-through sea water, diet, and possibly effects of early experience of mechanical stimulation must affect statoconium replication, especially directly after metamorphosis, and need to be investigated.

Schmidt (1912), and McClary (1963) have postulated intraluminal crystallization of stones from a $CaCO_2$-organic fluid matrix. This process may be interfered with by the laboratory culture conditions and thus directly limit statoconia production by preventing the crystallization process rather than interfering with the intracellular production of their components. However, in a recent study of field-collected adult *Hermissenda,* the evidence supported the hypothesis that statoconia production occurred intracellularly within large coated vesicles (Kuzirian *et al.*, 1981). Evidence for the intracellular production of statoliths was also reported by Geuze (1968) for the basomatophoran pulmonate *Lymnaea stagnalis.* Thus, the spurious intraluminal protein matrix is totally artifactual and probably directly related to culture conditions.

Previous studies have often utilized statocyst extirpation experiments as a means of assessing the contribution of the statocyst pathway to the orientation of marine invertebrates, especially crustaceans, in which the statocysts are often located in the bases of the easily removable antennules (Creutzberg, 1975). Similar experiments in a soft-bodied animal such as *Hermissenda,* in which the statocysts are in close contact with the cerebropleural ganglion and are only visible upon dissection, are not readily feasible. Therefore, the discovery that animals possessing either one, several, or a typical complement of statoconia could be reliably produced provides a useful tool for analyzing the contribution of the statocyst pathway to the behavioral change resulting from stimulation with paired light and rotation. The contribution of nonspecific stimulation resulting in arousal could not be expected to change in these preparations. Therefore, changes in the efficacy of the associative effect indicate that the statocyst pathway is important in mediating this example of associative learning.

The nature of mechanotransduction by the sensory cilia has been investigated in both *Hermissenda* (Alkon, 1975; DeFelice and Alkon, 1977; Grossman *et al.,* 1979; Stommel *et al.*, 1980; Grossman and Alkon, 1983) and *Aplysia* (Gallin and Wiederholt, 1977). Grossman *et al.* (1979) found that voltage noise variance of hair cells with cut axons increased with increasing depolarization when the isolated nervous system was rotated at accelerations from 0.5 to 2.0 *g*. Hair cells with unloaded hairs (opposite to the mass of statoconia) had low noise levels during rotation, although the hairs continued to beat. The authors concluded that both the voltage noise and the generator potential originate from contact between the statoconia and the motile cilia. Evidence from voltage noise power spectra recorded from *Hermissenda* hair cells also indicates that most noise arises from statoconia-ciliary interactions (Grossman and Alkon, 1983). Recordings from hair cells of a statocyst containing a single statoconium from a wild *Hermissenda* showed a smaller voltage noise variance than those from a typical statocyst (DeFelice and Alkon, 1977). The size of the generator potential can be considered to reflect the average value of all the conductance changes initiated as a function of stimulus strength, in turn a function of the number of statoconia as well as the acceleration of gravity (*g*-value) (DeFelice and Alkon, 1977).

Stommel *et al.* (1980) studied the mechanism of sensory transduction in the *Hermissenda* statocyst by modifying the pattern of ciliary beat. Intracellular injection of vanadate anion was followed by a decrease, then cessation of voltage noise paralleling progressive ciliary relaxation, then loss of motility. Injection of 4,4′-dithiobisphenylazide caused the cilia to stiffen, with an increase in voltage noise amplitude if the cilia were beating, and minimal noise when the beat was inhibited. The authors suggest that mechanical stimuli are transduced at the junction between the ciliary membrane and the plasma membrane, where a beating cilium causes flexing of the adjacent plasma membrane.

In intact *Hermissenda,* the less effective transduction of hair cells when only one statoconium is present provides a functional explanation for the significantly reduced effectiveness of training in these animals. Any factor, such as reduction of statoconium number, that decreases the effect of rotation on hair cell depolarization would also be expected to decrease the amount of inhibition received by the photoreceptors, whether directly through possible monosynaptic connections or by way of projections onto the optic ganglion cells.

Details of the circuitry by which a decrease in hair cell generator potential and spike frequency causes a decreased effectiveness of training with paired light and rotational stimulation have been proposed (see Fig. 7 and Tabata and Alkon, 1982). Because multiple pathways allow feedback from interneurons in the optic and cerebropleural ganglia onto hair and photoreceptor cells, we cannot rule out effects of activity in other circuits on acquisition and retention of behavioral conditioning. The only observable morphological variable in this study that might affect caudal hair cell function was reduction in statoconium number and reduced lumenal surface area. Based on present knowledge, it seems reasonable that as a result of decreased synaptic output due to less rotation-induced caudal hair cell stimulation, less depolarization of the type B photoreceptor follows pairings of light and rotation (Alkon, 1984).

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PARTICLE TRANSPORT AND INCORPORATION DURING SKELETON FORMATION IN A KERATOSE SPONGE: *DYSIDEA ETHERIA*

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ABSTRACT

The mechanism and significance of sand particle incorporation into skeletal fibers were investigated in the sponge, *Dysidea etheria.* Time lapse cinemicrography of the sponge surface showed that this species actively transported particles at an average rate of 7.5 μm/min to areas where skeletal fibers were formed. Transport was associated with specific structures of the sponge's dermal membrane. Sand particles applied to sponge explants for five weeks increased skeletal fiber growth over growth in explants experiencing ambient particle loads. Explants deprived of particles had significantly less fiber growth. The location of applied particles influenced the direction of fiber growth. The results indicate that particles enhance skeletal fiber growth by acting as a filler material in the construction of fibers and may influence the overall direction of growth and shape of the skeleton.

INTRODUCTION

Sponges build a wide variety of skeletal structures. Many demosponges supplement their spongin skeletons with particulate material acquired from the environment, and the regular presence of particles in the skeletal fibers of some sponges has been used as a taxonomic character in sponge identification (*e.g.,* Lendenfeld, 1889, de Laubenfels, 1950). The incorporation of macroscopic pieces of foreign material in a supporting structure is an unusual feature that appears to be unique to sponges. Other organisms, like polychaete worms, use sand to build protective tubes, but such structures are outside the body of the animal whereas the sponge's skeletal fibers are internal. Thus, unlike their use in the worm tube, the incorporation of particles during skeletogenesis in the sponge must be closely coordinated with the sponge's growth and morphogenesis.

Previous discussions of particle incorporation in sponges addressed mechanisms of particle selection (Schulze, 1879, Lendenfeld, 1889, Sollas, 1908) and functions of particles in support and skeletogenesis (Shaw, 1927). This work relied on the study of fixed material which did not allow direct observation of the processes of particle in-incorporation and skeletal growth. Although these studies clearly showed that particles were associated with skeletal fibers, sometimes in very regular patterns, little was known of the pathway of the particle as it moved from the environment into the skeletal fibers. In addition, the observed associations of particles with fibers did not unequivocally demonstrate their requirement or role in skeletogenesis (see *e.g.,* Shaw, 1927). The purpose of the present study was to elucidate the process of particle incorporation and to investigate the role of particle incorporation in skeletal growth. Time lapse

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Received 26 August 1985; accepted 19 January 1986.

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cinematography was used to directly observe the slow processes of particle sorting on the surface of the sponge that led to incorporation of particles into growing skeletal fibers. Field manipulations were performed to determine the effect of particle availability on sponge fiber growth.

MATERIALS AND METHODS

Organism of study

The sponge studied was the keratose demosponge, *Dysidea etheria* de Laubenfels (Order Dictyoceratida). This species builds a skeletal network of spongin fibers that can be filled with a wide variety of foreign particles. Particles are restricted mainly to the radially arranged primary skeletal fibers. Secondary skeletal fibers crosslink the primary fibers to form a skeletal meshwork. The apices of the primary fibers abut on the dermal membrane that forms the surface of the sponge and create cone-shaped protrusions on the surface called conules (Fig. 1). The loose construction and relative transparency of this sponge allows the direct observation of interactions between the outer surface and underlying distal skeletal elements in the living material. The present study focused on growth of the primary skeletal fibers at the conules.

Collection and filming of sponges

All observations on living material were performed at the Bermuda Biological Station. Live sponges were collected with their basal substratum intact to minimize disturbance to the animal and were maintained in a Plexiglas flow through tank (flow rate = 1 cm/s) to obtain as normal a behavior as possible. Sponges were filmed at one frame per minute with a 16 mm Bolex movie camera using Kodak Plus-X 7276 reversal film. The camera was mounted over the phototube of a Wild M5 stereomicroscope (field of view 9 by 11 mm) and sponges were filmed overnight for seven to nine hours under constant illumination of a fiber optics lamp. Calcium carbonate sand particles (average diameter 112 μm, range 67–201 μm) were sprinkled on the surfaces of the sponges and several frames were shot to note the initial location of particles before time lapse filming began. Seven sequences were filmed but only one 7.5-hour sequence was analyzed in detail.

Frame-by-frame analysis was performed using a NAC Inc. DF-16C projector. Particle movement was analyzed in five circular conule areas and five randomly chosen rectangular nonconule areas (Fig. 1). Directions and rates of movements of individual particles on the sponge surface were measured from tracings of particle outlines every ten frames (= ten minutes). Vectorial representations of particle movement were produced by connecting the centers of sequential particle outlines (see Fig. 3). Directions of movement were measured by the angles formed between the line segments and could vary from −180 degrees to the left to +180 degrees to the right, with a zero angle indicating no change in direction of movement. To test if directions of particle movements were random, observed particle movements were compared to expected movements of randomly directed particles (having equal probability of moving in any direction) by comparing the distributions of observed angle values to the expected distribution generated by random movement using the Chi-square goodness of fit test (Sokal and Rohlf, 1969). Rates of particle movement were estimated from linear regressions of particle movement with time. Particle position was measured as the distance between the particle and the conule apex.

Skeletal growth experiments

The skeletal growth experiments were performed for five weeks in Walsingham Pond, Bermuda. Explants and whole sponges were used in experimental treatments. Explant samples were made by cutting whole sponges into 1 cm^3 pieces and tying the pieces onto glass coverslips with sewing thread. The coverslips were mounted on Plexiglas holders and attached to metal racks. There were seven to ten explant replicates per treatment. Explants were allowed to heal in the field for one week before beginning experimental treatments. Siliceous sand, which was distinguishable from the experimental sand, was applied to all sponges two days before the experiment and was incorporated into the skeleton to serve as a common reference point from which skeletal growth was measured.

There were five manipulations of particle availability in the experiment: three particle addition treatments and two particle depletion treatments. In the addition treatments, calcium carbonate sand particles of three different sizes, obtained by crushing and sifting beach sand through a graded series of sieves, were applied to cleaned sponge surfaces every other day. The average diameters of the three size classes of sand were 112 μm (small), 250 μm (medium), and 540 μm (large). The white color of the carbonate sand differentiated it from indigenous Walsingham Pond sediments. The particle depletion treatments consisted of cages made of 100% nylon fabric (mesh size 40 μm) that enclosed the explants and excluded particles. Explants caged on alternate days (half caged) had 32% average dry weight of ambient particles excluded and explants kept constantly caged (fully caged) had 71% average dry weight of ambient particles excluded. Particle exclusion was assessed by collecting sediment samples in small plastic containers for three 5-day periods inside and outside of cages. Controls for all treatments were explants exposed to ambient particle loads. The secondary effects of cages were examined with half and fully caged explants exposed to medium particles and compared to uncaged explants treated with medium particles.

The effect of the location of particle application on skeletal growth was observed in three whole sponges that were turned on their sides prior to application of medium particles. Three control sponges were similarly reoriented and grown under ambient particle conditions.

At the end of the experiment, samples were collected, fixed for 2 days in 10% formalin with 0.5% CPC (cetylpyridinium chloride) to aid matrix preservation (Williams and Jackson, 1956) and transferred to 70% ethanol for storage. Samples were stained with saturated basic fuchsin in 95% ethanol, embedded in paraffin, and sliced into approximately 5-mm thick slabs. The slabs were deparaffinized and embedded in Caroplastic (Carolina Biological Supply Co.) which polymerized to a colorless material of high refractive index and allowed observation of the sponge skeletal fibers. Lengthwise growth of primary fibers was measured using a Wild M5 stereomicroscope. Fibers from sponges not treated with carbonate particles were measured from the intercalation of the siliceous marker sand. Fibers from sponges treated with particles were measured more easily from the intercalation of the more numerous carbonate particles. This point approximately coincided with the position of the siliceous sand.

The mean fiber length for each explant constituted an independent observation in one-way analyses of variance (ANOVA) for the effects of the treatments on fiber growth. Separate analyses were made for particle additions, particle depletions and cage effects. Multiple *t*-tests were performed when the ANOVA treatment effect gave a significant result. The Bonferroni inequality that adjusts confidence levels for multiple comparisons was applied to multiple *t*-tests. All statistical procedures were performed in SAS (Statistical Analysis System, 1982).

RESULTS

Dermal membrane morphology and behavior

Figure 1 shows the morphology of the filmed area of the sponge. The dermal membrane of *D. etheria* is characterized by a reticulating pattern formed by thicker regions of the dermal membrane mesohyl. Radially oriented reticulations converge upon the raised conule apices where the primary skeletal fibers meet the dermal membrane; connecting reticulations between conules lack such orientation. The areas between reticulation lines in the dermal membrane are perforated by groups of ostia. Excurrent oscula arise from nonreticulated, smooth areas of the membrane.

Time lapse cinemicrography showed numerous dermal membrane contractions and changes in the appearance of reticulations. The contractions gave the membrane an oscillating appearance and contributed to particle movement. Reticulations were not initially distinguishable but gradually became more distinct about sixty minutes after particle application. Evidence of membrane contractions and changes in reticulation appearance were also observed in nonfilmed sponges.

Particle movement

Initially, particles were scattered randomly over the entire membrane surface. Within thirty minutes after application, most of the particles on conule areas had moved down the slopes of the conule sides, presumably by gravity, and collected at the bases of the conules (Fig. 2a). As the dermal membrane reticulations became more visible, scattered groups of two to ten particles aggregated into larger clumps and became aligned on the reticulations (Fig. 2b). The particle clumps could be maintained or could disaggregate during subsequent movement.

Slow movement of particle clumps was observed on reticulation lines after particle aggregation and alignment. Most movement was observed on the radial reticulations in conule areas, where particles moved from the base to the apex of the conule (Figs. 2b–e, 3). The particles became harder to distinguish as they neared the apex area, probably as a result of their engulfment into deeper layers of the sponge (Fig. 2f). Limited particle movement was also observed on connecting reticulations in nonconule areas but was more difficult to discern since particles often stopped at branch points. The diffuse branching pattern also made the net direction of movement more difficult to determine. Approximately 20% of the particles applied to the sponge moved slowly. Nearly all slow movement ended 200 minutes after particle application.

The slow movements of particles were easily differentiated from more rapid and erratic movements of particles caused by contractions of the dermal membrane (Figs. 2d, 3). Slow movement of particles maintained fairly constant rates averaging 7.5 ± 2.1 μm/minute (n = 15) (Fig. 4) and particles moved nonrandomly towards conule apices, as shown by comparison of the direction of movement with a random movement pattern (Fig. 5). By contrast, erratic movement of particles could occur more quickly (maximum rate 27.9 ± 5.5 μm/min, n = 4), was sustained for shorter periods of time (Figs. 2d, 4) and was random (Fig. 5). Henceforth, the slow movement of particles will be referred to as transport.

Not all particles stayed on the sponge surface. Occasional rapid disappearances could be detected on film and attributed to inhalation of particles. General observations of particle-treated sponges indicated that particles could be consolidated in mucus clumps and sloughed off the sponge surface.

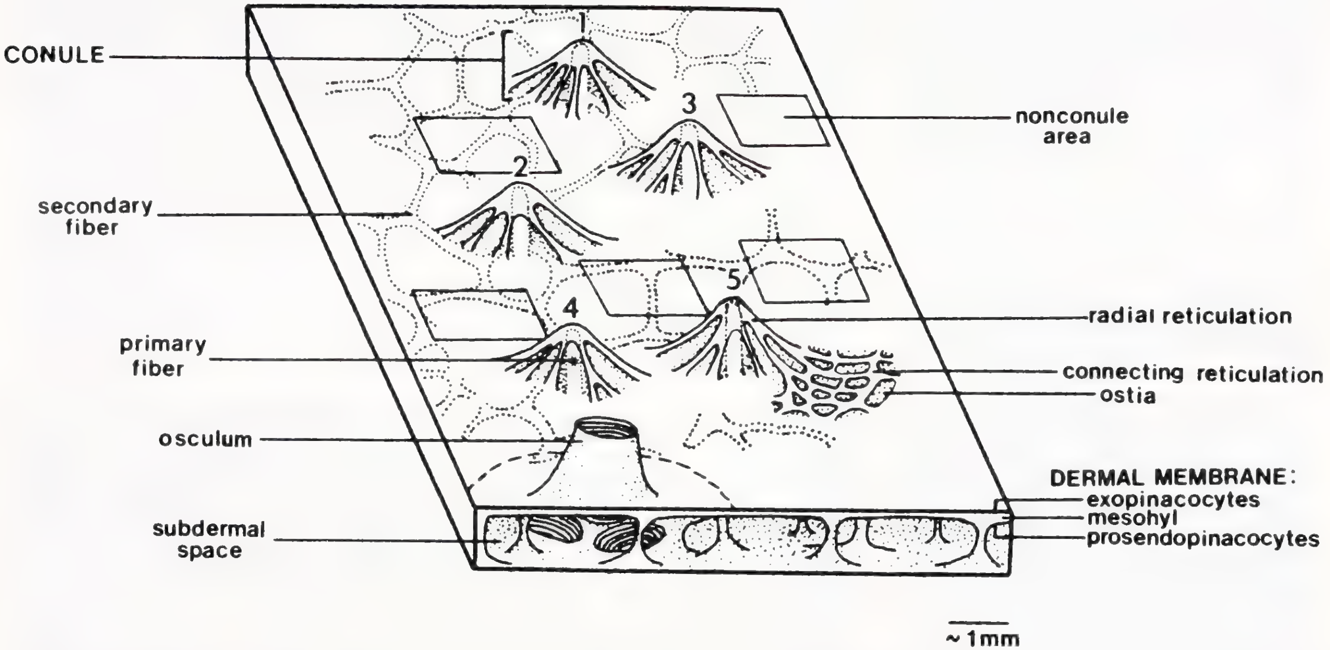
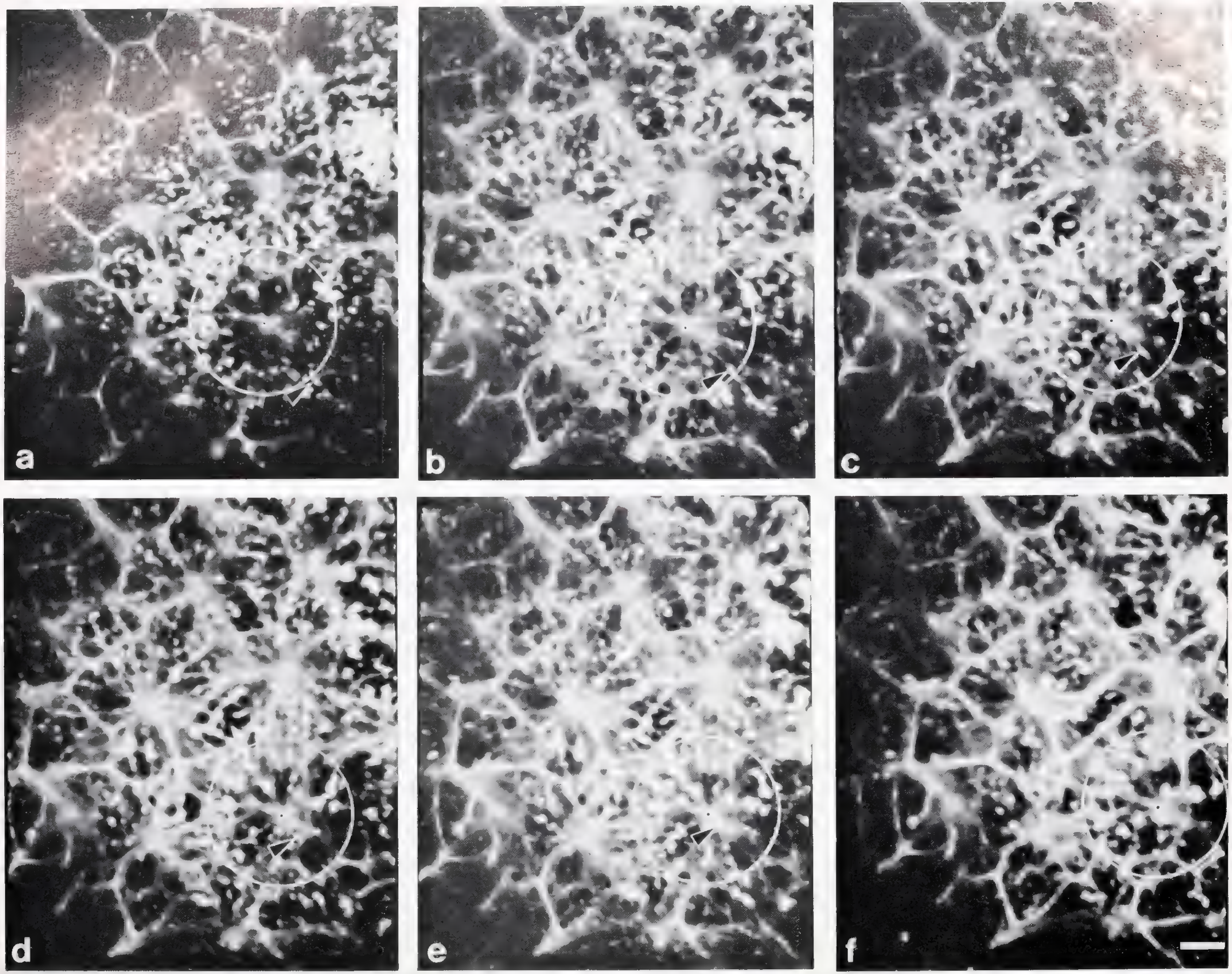

CONULE
secondary fiber
primary fiber
osculum
subdermal space
1
2
3
4
5
nonconule area
radial reticulation
connecting reticulation
ostia
DERMAL MEMBRANE:
exopinacocytes
mesohyl
prosendopinacocytes
~1mm

FIGURE 1. Diagram of the sponge surface observed in the film. Note three cell compartments that form the outer dermal membrane of the sponge. Below the dermal membrane is the water-filled subdermal space. The dermal membrane is raised by primary skeletal fibers to form projections on the surface called conules. Secondary skeletal fibers crosslink primary fibers within the sponge interior (skeletal elements indicated by stippled lines). Mesohyl cells form reticulating patterns in the membrane except in areas around oscula (dashed line). Shown are the five conule areas (numbered) and five nonconule areas (rectangles) observed for particle behavior.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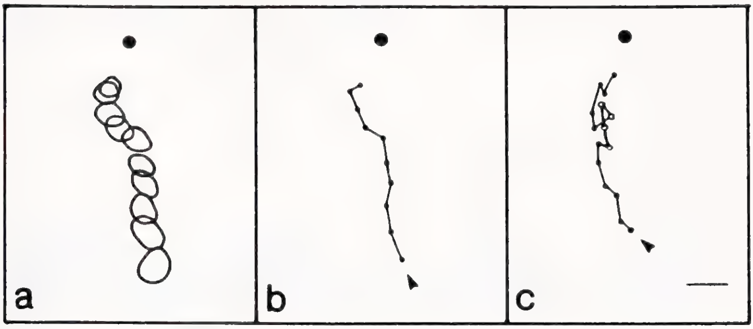

FIGURE 3. Tracings of slow and erratic movements of particles. Shown are two examples of particles moving up radial reticulations to a conule apex (dot), segments equal 10 minutes. (a) Outlines of a moving particle. (b) Linear representation of particle in (a), all movement slow transport. (c) Linear representation of a different particle showing transport interrupted by erratic movement due to dermal membrane contractions (open circles). Scale = 200 μm.

Effects of particles on skeletogenesis

The effects of particle addition and depletion treatments are shown in Figure 6. The addition of particles to explants produced a significant increase in primary fiber length over ambient lengths (Fig. 6a, Table I, $P = .0484$). Although Figure 6a shows a trend of increasing fiber length with increasing particle size, Bonferroni adjusted pairwise *t*-tests showed no significant differences between any two addition treatments (Table I). The effect of depletion of particles on fiber length was also significant (Fig. 6b, Table II, $P = .0004$) and *t*-tests showed that both half caged and fully caged treatments significantly reduced fiber growth (Table II). While cages undoubtedly affected flow rates and light penetration to explants, they did not significantly slow growth of primary fibers (Fig. 6c, Table III, $P = .4682$). Dye releases inside the caged treatments in the field demonstrated the presence of a slow flow which evidently was sufficient to sustain sponges at near normal rates of growth.

In reoriented whole sponges that had been turned on their sides, primary fiber growth gradually became deflected upwards, perpendicular to the former vertical axis of the sponge (Fig. 7). The reoriented fibers were continuous with pre-existing fibers. These whole sponges showed substantial growth of primary fibers up to three times greater than growth in explanted sponges (15 mm in comparison to 5 mm). The increase in primary fiber length created an average percent increase in whole sponge volume estimated at 40% from measurements of Caroplast slabs. Reoriented control sponges subject to ambient particle levels had less upward reorientation of primary fibers, less overall fiber growth, and increased in volume by 26%.

DISCUSSION

Interactions of particles with the sponge surface

The incorporation of particles into sponge skeletons has long been a subject of debate (Schulze, 1879, Lendenfeld, 1889). There was general agreement that sponges

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FIGURE 2. Time lapse cinemicrography of particle transport. Movement of a particle on conule 5 shown by the arrow in the circular area; black dot indicates the conule apex. All times are elapsed time after particle application. (a) 25 minutes, dispersed particles clustered at bases of conules. (b) 50 minutes, particle clumping. (c) 90 minutes, slow transport of particle. (d) 97 minutes, rapid displacement of particle by membrane contraction. (e) 150 minutes, continuation of slow transport. (f) 370 minutes, particle has been engulfed in vicinity of apex and is not clearly visible. Scale bar = 1 mm.

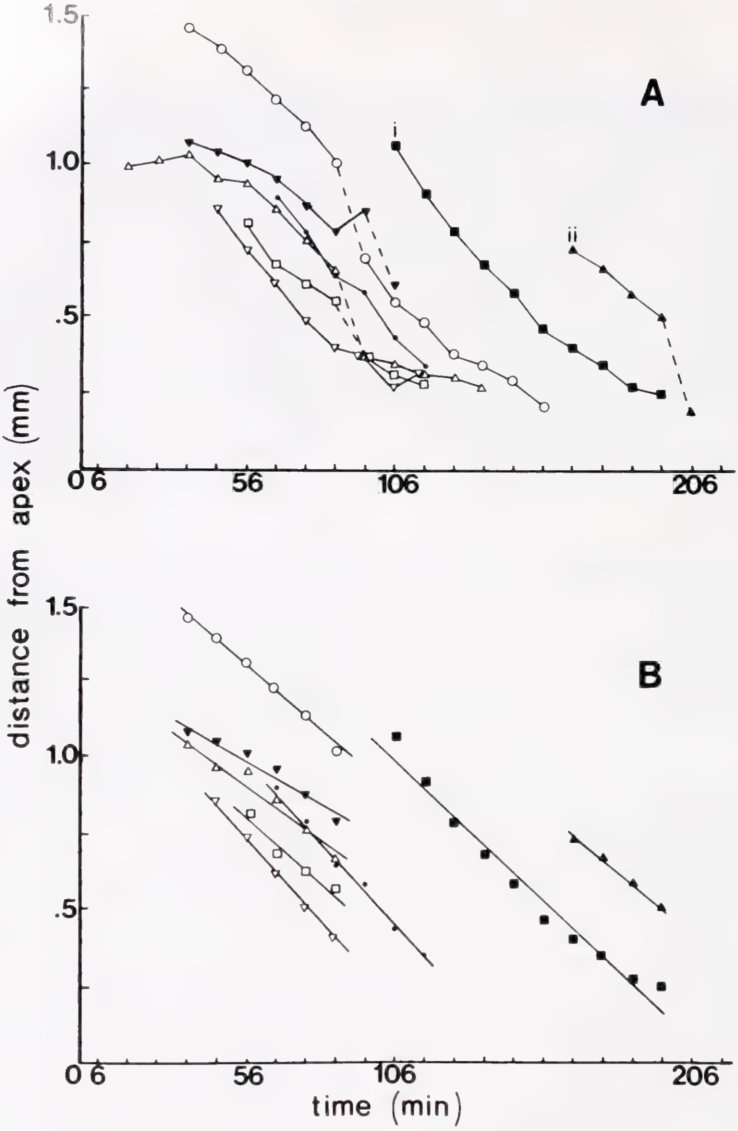

FIGURE 4. Plots of positions of eight particles on conule 5. All particles are moving linearly towards the conule apex (see Fig. 3). (A) Dashed lines indicate erratic movement interrupting slow transport; these were omitted from calculations of transport rate. Note late movement of particles (i) and (ii). (B) Examples of linear regressions to calculate slow transport rate fitted to selected portions of plots in (A).

could select particles since some species appeared to incorporate only foreign spicules in their fibers, but there was disagreement on the mechanism of particle selection. Schulze (1879) suggested that particles were mechanically sorted by physical interactions of the sponge surface with its environment, such as the interaction between surface stickiness and water flow. He suggested that differences in the physical characteristics of sponge surfaces would influence the type of particles incorporated, akin to the deposition of different types of sediments in different parts of a river bed.

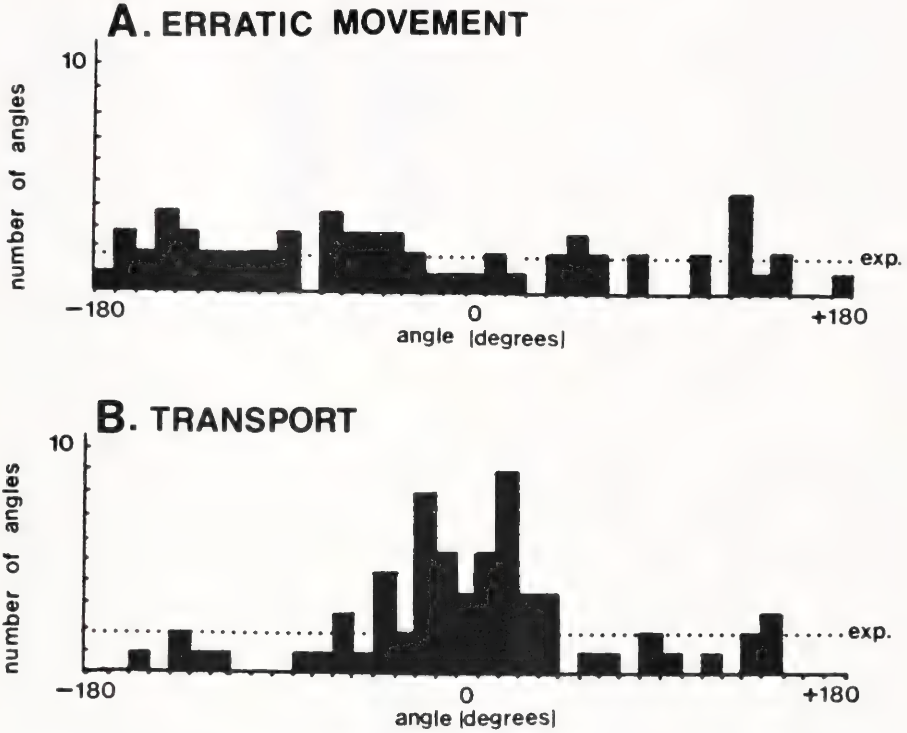

FIGURE 5. Distribution of turning angles for transported and erratic particle movements. (A) Direction of movement for 10 erratic particles (65 angles measured). Expected frequency per angle = 1.76 and indicated by dotted line; $\chi^2 = 34.61$, d.f. = 36, $P < .25$, movement not significantly different from random. (B) Direction of movement for 11 transported particles (72 angles measured). Expected frequency = 1.95, $\chi^2 = 118.14$, d.f. = 36, $P < .001$, movement significantly different from random.

Lendenfeld (1889) argued for a more active selection of particles by the sponge since he observed that a species always incorporated the same kind of foreign material in its fibers, regardless of differences in habitat distribution of individual specimens. Schulze's sorting mechanism insufficiently explained such observations and implied that the nature of particles incorporated into the sponge could change with changing environmental circumstances. However, Lendenfeld's observations lack complete documentation since he did not compare local environmental particle compositions with the composition of particles incorporated in the sponges living in each locality. In addition, these and other studies only speculated on the detailed mechanisms of particle incorporation since they were based on static observations of the particle composition of preserved sponges.

The time lapse observations of the present study support Lendenfeld's views on active selection of particles and provide the first information on the mechanism of particle incorporation by demonstrating the presence of an organized particle transport system on the surface of *D. etheria*. The pattern of transport of particles is correlated with the pattern of reticulations in the dermal membrane but more needs to be known about the factors that determine reticulation patterns and direction of transport on reticulations. Histological studies show that cells of the dermal membrane mesohyl interact with particles and may migrate to transport particles, so regulation of directions of cell migration may determine particle transport patterns (Teragawa, 1985).

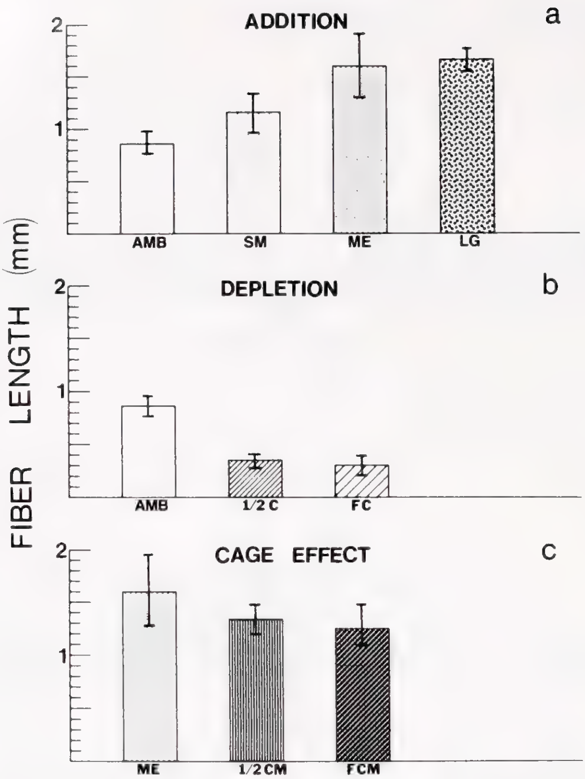

FIGURE 6. Effect of particle availability on primary fiber growth. Mean fiber lengths and associated standard errors of the mean shown for all treatments. (a) Fiber growth with particle additions. AMB = ambient control, SM = small particles, ME = medium particles, LG = large particles. (b) Fiber growth with particle depletions. AMB = ambient control, ½ C = half covered, FC = fully covered. (c) Fiber growth and cage effect. ME = medium particles (uncovered), ½ CM = half covered plus medium particles, FCM = fully covered plus medium particles.

Particle transport is probably only one part of the sponge's response to particle loads. Occasionally, the sponge is likely to receive more particles than it can use, as was the case in experimental manipulations. Not all particles applied were transported and the sponge must be able to clean its surface of excess particles to prevent fouling

TABLE I

One-way analysis of variance of the effect of particle addition treatments on primary fiber length

Source	Sum of sq.	d.f.	Mean square	F	P
Particle additions	2.454	3	0.8181	3.08	.0484
Error	5.838	22	0.2654		
Total	8.292	25			
Treatment groups:			AMB SM ME LG[1]		

[1] Bar indicates homogeneous subgroups differentiated by Bonferroni multiple comparison tests; abbreviations as in Figure 6.

TABLE II

One-way analysis of variance of the effect of particle exclusion treatments on primary fiber length

Source	Sum of sq.	d.f.	Mean square	F	*P*
Particle exclusion	1.1239	2	0.5619	12.20	.0004
Error	0.8750	19	0.4605		
Total	1.9989	21			
Treatment groups:			AMB 1/2 C FC[1]		

[1] Bar indicates homogeneous subgroups differentiated by Bonferroni multiple comparison tests; abbreviations as in Figure 6.

by other organisms and to allow efficient water exchange. Particle-using sponges like *D. etheria* may thus possess dual and somewhat contradictory responses to particle loads: to retain some particles for use in the skeleton and to eliminate excess particles that cannot be incorporated. Behaviors like the oscillating contractions of the dermal membrane observed in the time lapse filming and the sponge's pumping activities may contribute to elimination of particles.

A summary of the sponge's behavioral responses to a single application of particles can be reconstructed from the time lapse movie results and general observations of particles on sponges (Fig. 8). Percentages were estimated from direct counts of particles in conule and nonconule areas and probably represent maximum values due to the abnormal overloading of the sponge surface with particles. As shown, some particles can be quickly removed from the surface by inhalation through ostia. Other particles retained on the surface for longer time periods are sorted into three groups: particles that are slowly moved off the surface by transport, dermal membrane oscillations, or mucus sloughing; particles that become transported to skeletal fibers; and particles that are directly engulfed without transport. Transport and incorporation of particles into primary fibers are the slowest processes in the sorting of particles on the sponge surface and direct engulfment of particles occurs when these processes are overloaded. Engulfed particles may become incorporated into secondary skeletal fibers forming near the surface since heavily loaded sponges in field growth experiments often had secondary fibers filled with particles (Fig. 7). In normal *D. etheria* skeletons, secondary fibers sometimes contain particles and these may represent particles directly engulfed during periods of heavy particle loads.

TABLE III

One-way analysis of variance of the effect of cage treatments on primary fiber growth

Source	Sum of sq.	d.f.	Mean square	F	*P*
Cage effect	0.4517	2	0.2258	0.78	.4682
Error	6.6228	23	0.2879		
Total	7.0745	25			
Treatment groups:			ME 1/2 CM FCM[1]		

[1] Bar indicates homogeneous subgroups differentiated by Bonferroni multiple comparison tests; abbreviations as in Figure 6.

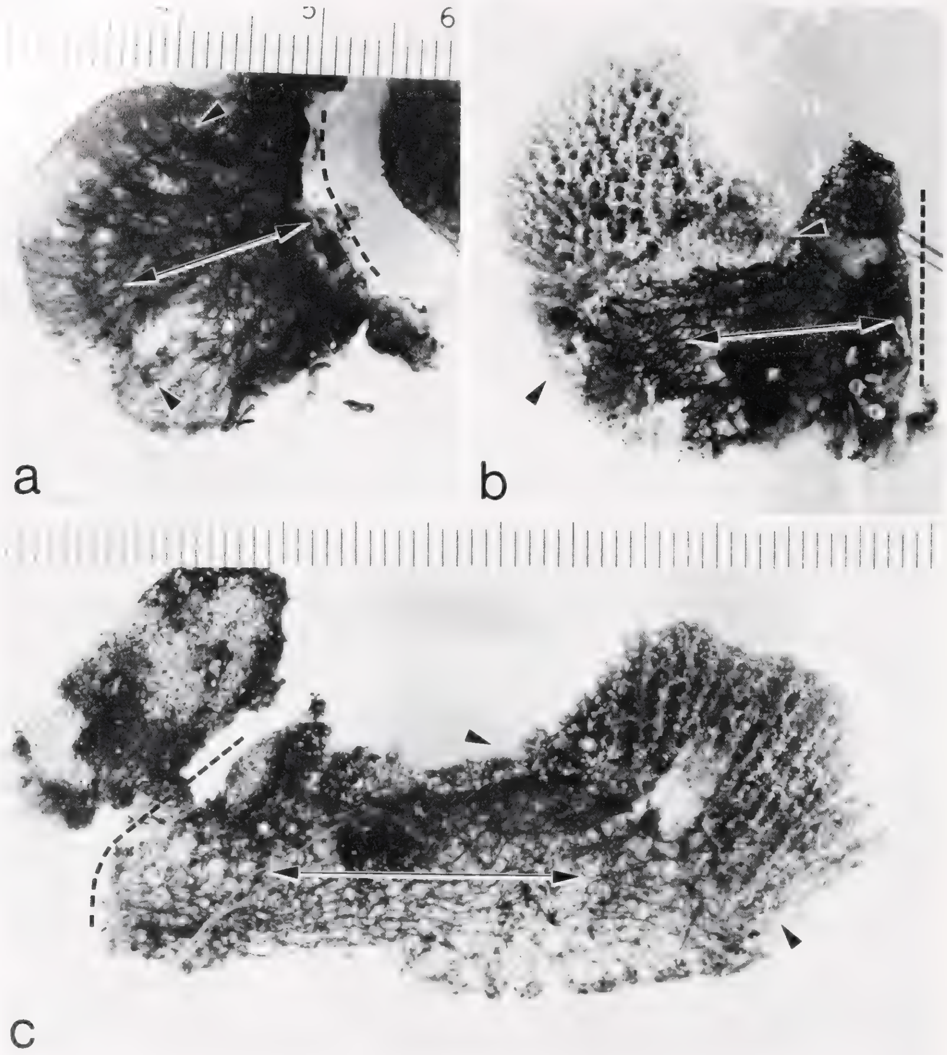

FIGURE 7. Effects of particle treatments on skeletal form. Caroplast sections shown in approximate reoriented positions. Dashed line indicates base of sponge, double arrow indicates original vertical axis. Start of new growth indicated by arrowheads. (a) Cross-section through a reoriented control sponge under ambient particle loads. Arrowheads indicate line of siliceous marker sand. Fiber growth is less and fibers not as strongly reoriented as in treatment sponges. (b and c) Sections through reoriented sponges treated with medium particles. Note how primary fibers smoothly bend upwards; white particles fill the primary and secondary fibers. Scales in mm.

Effects of particles on skeleton growth

The results from the particle addition and depletion experiments on explants show that primary fiber growth can vary with particle availability (Fig. 6). The observations suggest that particles serve as a filler material in the skeleton, allowing the sponge to

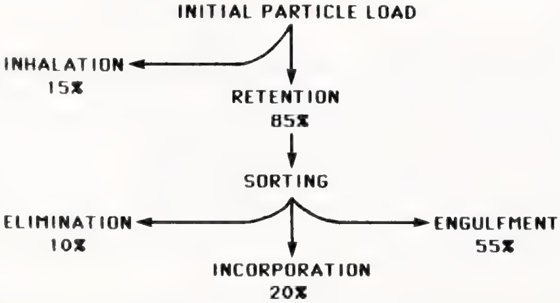

FIGURE 8. Proposed sequence of surface/particle interactions for a single particle application. Percentages are estimated from time lapse film. Smaller particles can be eliminated from the surface by inhalation, remaining particles are sorted on the surface and eliminated, incorporated into skeletal fibers, or directly engulfed into the surface. See text for details.

secrete less material to construct skeletal fibers. Although the correlation between fiber growth and particle size was not found to be statistically significant, the trend of the data suggests that large particles allow a greater increase in fiber length than do small particles, presumably because large particles occupy more fiber volume.

Comparisons of explant and whole sponge growth suggest that the upper limit to fiber growth rate may be controlled by the size of the sponge, since fibers measured from the smaller explants grew less than fibers of the larger, reoriented whole sponges. Larger whole sponges also grew more than smaller whole sponges. A larger sponge may be able to recruit a larger population of spongin-secreting cells to areas of fiber growth due to the sponge's proportionately greater volume, resulting in increased secretion of spongin to consolidate more particles.

Particles were never completely eliminated from the surfaces of sponges in this study, thus the demonstration of an absolute requirement for particles in fiber growth was not possible. However, fiber growth in particle-depleted treatments was significantly decreased to less than half the rate of ambient samples, suggesting that particles have an important role in normal skeletogenesis. No specimen of *D. etheria* was observed to have primary fibers devoid of particles. The incorporation of particles during skeletogenesis may enhance growth rates to allow *D. etheria* to successfully compete for space in the field.

The reorientation of primary fiber growth in reoriented whole sponges may have been caused by a general effect of gravity or light on the sponge or by the specific change in the direction of particle loading. The effects of these parameters could not be clearly separated in the field. However, control sponges exposed to ambient particle loads had fibers that were not as strongly reoriented as fibers of the particle treated sponges (Fig. 7), suggesting that particle load was responsible for the reorientation of primary fiber growth. If gravity or light was the sole influence on fiber orientation, one would expect to see the same amount of fiber deflection in control and treatment sponges, unless reorientation was a function of the amount of growth which differed between the control and treatment sponges. Reorientation of primary fiber growth may be due to skewed patterns of particle transport to primary fibers.

At present it would be premature to generalize about mechanisms of particle incorporation from the example of *D. etheria* alone. Sponges more selective than *D. etheria* in their choice of particles probably have different mechanisms of particle incorporation. More comparative and quantitative studies are necessary to establish the general nature of the transport patterns observed here.

ACKNOWLEDGMENTS

I thank H. F. Nijhout, S. A. Wainwright, H. Erickson, D. R. McClay, S. Vogel, [illegible]ias, R. Harris, P. C. Wainwright, M. R. Truschel, B. Woodbury, and P. [illegible]bury for their assistance through various stages of this project. I also thank the staff of the Bermuda Biological Station for their generous assistance over several summers. This research was supported by funds from the Cocos Foundation, an Exxon Fellowship administered through the B.B.S., the Lerner-Gray Fund for Marine Research, Sigma-Xi, and the Duke University Graduate School. This is contribution number 1079 from the Bermuda Biological Station.

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A COMPARATIVE STUDY OF THORACIC AND CHELIPED MUSCLE ASYMMETRY IN MALE FIDDLER CRABS (GENUS: *UCA*)

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ABSTRACT

Asymmetries in thoracic and leg muscles that move the single huge major cheliped of male fiddler crabs are compared to their contralateral homologues in nine species of *Uca*. In *Minuca* (*U. longisignalis, U. minax, U. pugnax,* and *U. rapax*) and *Boboruca* (*U. thayeri*) species, the major side body muscles are twice as large as the minor muscles, with more muscle fibers. Additionally, the individual sarcomeres are exceptionally long, reaching over 20 μm in length. In contrast, in species of the sub-genus *Celuca* (*U. crenulata, U. panacea, U. pugilator,* and *U. speciosa*), while these muscles may be up to six times larger than their contralateral homologues, with fibers nearly twice as long, the sarcomeres are not so hypertrophied.

Major muscles in the chela can be 20 times larger than their contralateral homologues. The largest chela muscles also show exceptionally large sarcomeres among *Minuca,* but not in *Celuca.*

It is shown that sarcomere lengths are inversely correlated with the speed and duration of the courtship wave behavior.

INTRODUCTION

Investigations of neuromuscular mechanisms associated with motor activities in animals have demonstrated the correlations between contractile fiber types, motor innervation, and their functional role in behavior. Crustaceans are especially favorable material for such studies due to the parsimony of their motor innervation (Atwood, 1972, 1973). Additionally, in some crustaceans the first pair of thoracic chelipeds, or claws, are bilaterally asymmetrical, consisting of a minor or relatively unspecialized chela and a major or more specialized contralateral chela. The neuromuscular basis of such asymmetry in the paired claws have been extensively studied in lobsters (review by Govind, 1984) and snapping shrimps (review by Mellon, 1981) and such studies provide fertile material for correlating neuromuscular specializations with behavior.

Perhaps the most flamboyant example of claw asymmetry is found in male fiddler crabs (Ocypodidae: genus *Uca*) in which the major claw is up to 20 times larger than the minor claw and may weigh almost as much as the rest of the animal. This major claw is used primarily for sexual and agonistic displays and interactions. Stereotypic waving (Crane, 1957, 1975; Salmon and Atsaides, 1968a, b), or sound production (Salmon, 1965, 1967; Salmon and Atsaides, 1969) by the major chela plays an important role in sexual behavior and in maintaining reproductive isolation (Salmon *et al.,* 1978), at least in some sub-genera of *Uca* (see Salmon, 1984). The major chela muscles, including those in the thorax, would be expected to show specialization as

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Received 22 November 1985; accepted 19 January 1986.

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Non-standard abbreviations: CL: carapace length; L_P = posterior levator muscle; ML: manus length; R: range; SD: standard deviation.

compared to those of the minor chela of the male or to the symmetric small chelae of the female. Since these display waves and acoustic signals are species-specific, comparisons between species of *Uca* provide valuable material to determine whether such behavioral differences between closely related species can be correlated with differences in the morphological and physiological characteristics of the muscles responsible for the behavior.

In fiddler crabs, knowledge of the mechanisms underlying the ontogeny of chela asymmetry is still incomplete. In the first post-larval crab stages the chelae are small and symmetrical (Morgan, 1923). In many species handedness is determined by chance at an early post-larval stage (Morgan, 1924; Vernberg and Costlow, 1966, Feest, 1969; Yamaguchi, 1977), as in lobsters, while in others right handedness is specified (Yamaguchi, 1977; Barnwell, 1982). At the muscle fiber level, asymmetry occurs early in post-larval life in *U. pugnax,* coincident with the first sign of chela hypertrophy (Trinkaus-Randall and Govind, 1985). Asymmetry in *Uca* is irreversible once it is established.

This paper reports fiber characteristics of muscles of the chelae of seven species of North American fiddler crabs, representing three of the seven sub-genera of *Uca* established by Crane (1975). It is shown here that sarcomere lengths (as estimated by A-band measurements) of certain muscles that move the fiddler crab major chela are inversely correlated with the speed and duration of the courtship wave display. Furthermore, certain fiddler crab chela muscles, and especially those from members of the sub-genus *Minuca,* show exceptionally large sarcomeres (*e.g.,* over 20 μm in *U. minax*).

MATERIALS AND METHODS

Animals

Species examined here and collection sites are as follows: *Uca (Minuca) minax,* Poquosin, Virginia and Fort McCalister State Park, Georgia; *U. (Minuca) pugnax,* Poquosin, Virginia; *U. (Minuca) rapax,* Sugarloaf Key, Florida; *U. (Minuca) longisignalis* (Salmon and Atsaides, 1968a) Panacea, Florida; *U. (Celuca) pugilator,* Panacea, Florida; *U. (Celuca) speciosa,* Big Torch Key, Florida; *U. (Boboruca) thayeri,* Fort Myers Beach, Florida. Several other species were examined less extensively: *U. (Celuca) panacea* (Novak and Salmon, 1974), Panacea, Florida, and *U. (Celuca) crenulenta,* Southern California. All of the crabs used were in intermolt (*circa* Drach stage C_4).

Experimental procedures

Animals were cold anesthetized (30 min on ice) and measurements of carapace length (CL), carapace width, and manus length (ML), from the heel of the propus to the tip of the pollex, were made with a vernier caliper (±0.1 mm). The side of the major claw, right or left, was also noted. The carapace was removed and the animal was immersed in seawater (25‰) chilled to 0°C, where removal of viscera and connective tissue was completed. The major and minor claws were secured in positions effecting unstressed extension of the muscles to be examined. For levators and remotors the claws were pinned forward and down, for promotors and depressors the claws were secured upward and back. For limb muscles the claw was secured either in the completely flexed position for stretcher and extensor muscles, or in the completely extended position for bender and flexor muscles. FAA fixative chilled to 0°C, a mixture of 30% ethanol, 7% formalin, and 3% acetic acid in water, was first injected with a hypodermic syringe into the region of the muscles, followed by immersion of the entire preparation in fixative for 8–12 h. After transfer to 30% ethanol, muscles were removed to water. Whole muscle weights of thoroughly blotted muscles were made with a Cahn model RTL electrobalance. To obtain muscle "fibers," muscles were teased apart with sharpened watchmakers' forceps. In this study the term "fiber" refers to a relatively discrete bundle of fibrils; these may or may not have been muscle fibers derived from individual cells. Such fibers were mounted on glass microscope slides in a mounting medium (7 g gelatin and 50 ml glycerol in 50 ml water).

Light microscopy

Measurements were made of A-band lengths rather than of entire sarcomeres, as the former are less affected by moderate stretch or contraction (Franzini-Armstrong, 1970). These measurements were made for each fiber with an optical micrometer using phase contrast optics (25× ocular; 100× Zeiss Neofluor oil immersion objective). The slide was moved on the microscope stage until the fiber to be measured was centered in the illuminated beam from the condenser; the objective was then rotated into position and the first discrete A-band encountered was chosen for measurement. This measurement, close to the center of the fiber, was taken as the estimate for that fiber. For thoracic muscles such an estimate was made for every fiber, but for the larger extensor and stretcher limb muscles A-band measurements were taken for every fifth mounted fiber. To prevent inadvertent bias slides were labeled with numbers from a random number table and then mixed, before measurements were taken.

Variability in A-band length within muscle fibers (Franzini-Armstrong, 1970) was determined by measuring a series of A-bands in single fibers (see Results, *Variability of muscle fibers,* below). It was quantified by using the Coefficient of Variation (CV), the standard deviation (SD) divided by the mean ($\bar{X}$) times 100. The statistical significance of differences in means, for A-band lengths of muscles of the major side compared to their minor side homologues, was determined using the one-sided student's *t*-test. The Spearman Rank Correlation Coefficient (rs) was used to test correlations of wave duration and A-band lengths in different species (Siegel, 1956).

In those cases where sarcomere lengths were used, they were measured similarly with a Zeiss Neofluor 40× objective and 25× ocular. The length of a row of five consecutive sarcomeres was measured. The average length was taken as the estimate of sarcomere length for the fiber.

RESULTS

Thoracic muscles of the cheliped segment

The thoracic musculature of two representative *Uca* species is shown from the dorsal side in Figure 1, with the major chelae on the left side in both cases. The four main thoracic cheliped muscles, the levator/depressor pair and the promotor/remotor pair, move the chelae up/down and forward/backward respectively. They are the primary thoracic muscles that move the major chela in the display wave. Arrangement of the muscles and skeletal elements are similar to those described for the portunid crab, *Callinectes sapidus* (Cochran, 1935).

The species of fiddler crabs examined could be divided into two groups according to the relative size and arrangement of the muscles of the major side. One group, species of the sub-genus *Celuca,* is exemplified by *U. pugilator.* The other group, represented by *U. minax,* includes all of the other sub-genera examined.

Table I shows the differences in weight between the major and minor side thoracic muscles of five individual crabs, and the weight ratios between the two sides. The

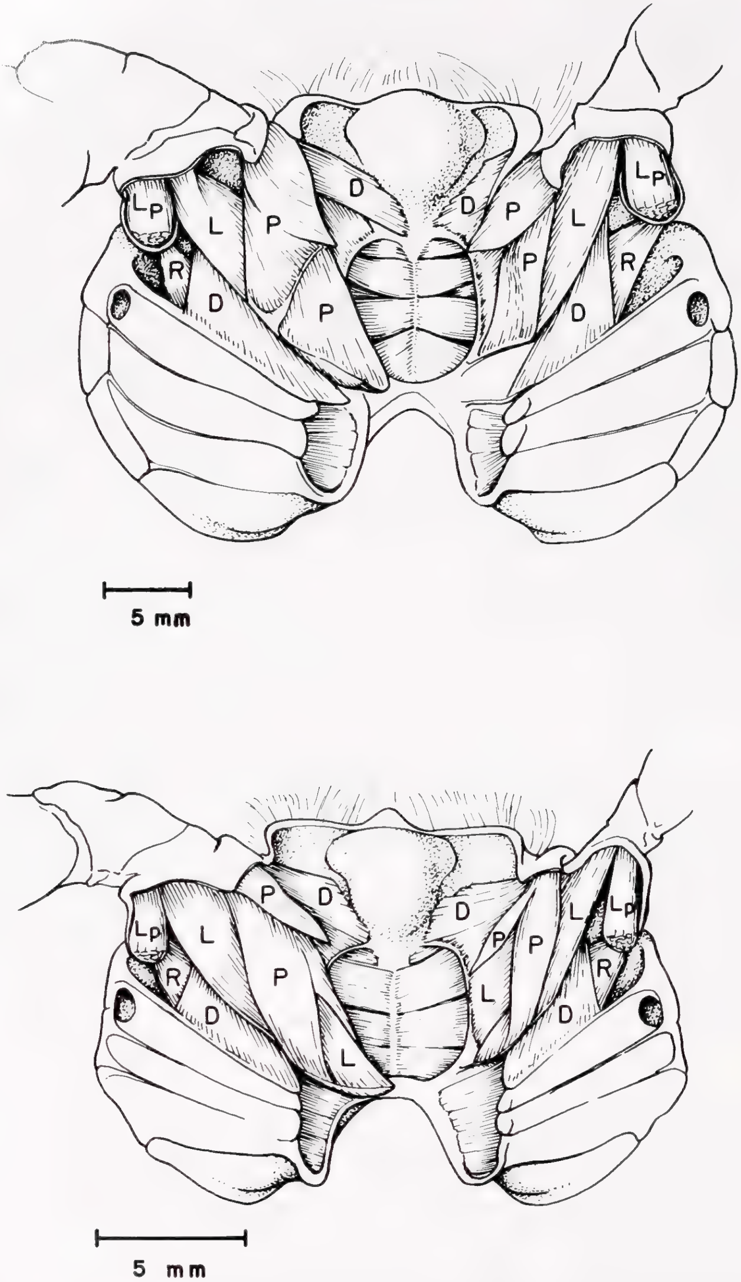
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FIGURE 1. Muscles of the cheliped segment in adult male *Uca minax* (upper figure) and *U. pugilator* (lower figure), viewed from the dorsal side after removal of the dorsal carapace and viscera. The major chela is on the left in both drawings. Muscles: Promotor (P), Levator (L), Posterior Branch of Levator (Lp), Remotor (R) and Depressor (D).

mean of the major to minor side weight-ratios for the two *U. pugilator* levator muscles, 6.43, is significantly greater than the equivalent mean ratio for two *U. minax* and one *U. pugnax*, 2.78. But with the exception of this larger size, of variable extent for the different thoracic chela muscles, and consequently a more posterior extension of muscle attachments, the muscles of the major sides resemble those of the minor sides in all of the animals examined.

Measurements of A-band lengths taken from mounted thoracic chela muscle fibers also show differences between members of the subgenera *Celuca* and *Minuca* as well as differences between the major and minor sides. The data is most complete for fibers from the levator muscle. Figures 2 and 3 show data for males of seven species, as well as from a single female *U. minax*. With the exception of *U. pugilator* these typify the dramatic differences ($P < 0.005$) in fiber composition between the major and minor levator muscles seen for the 20 individual males where data were obtained from both sides, or between male major and female chelae from *U. minax*.

The representatives of the subgenera *Minuca* and *Boboruca* in Figure 2 possess fibers with exceptionally long A-bands. *U. minax*, in particular, has many fibers with A-bands in the range of 12–14 μm. These would correspond to resting sarcomere lengths of 20 μm or more (Fig. 4), among the longest sarcomeres recorded from striated skeletal muscle in any animal.

The relationships between major and minor levators of *U. pugilator* (sub-genus *Celuca*) are variable. A-band distributions for three representative males (Fig. 3) show means and distributions for the major levators that are very similar, but by comparison the means of the minor levators are inconsistent among individuals. In one individual *U. pugilator*, the lower figure, the minor levator muscle has a greater mean A-band length than that of the major levator. In only one of these three examples (the middle figure in Fig. 3, left) was the difference between mean A-band lengths of major and minor levators significant. Major and minor levator muscles from seven other male *U. pugilator* showed similar variable relationships between the muscles of the major and minor chelae. The other species of *Celuca*, *U. speciosa*, shown in Figure 3, right,

TABLE I

Weights (mg) of major side and minor side thoracic muscles which manipulate the chelae, from five individual Uca

		Major/minor thorax muscle weights (major/minor ratio)			
Species	CL	Levator	Depressor	Promotor	Remotor
U. minax	20.7	27.8/10.5 (2.7)	24.9/12.0 (2.1)	22.4/11.2 (2.0)	22.3/8.4 (2.7)
U. minax	19.9	26.2/8.5 (3.1)	15.4/10.4 (1.5)	14.6/6.6 (2.2)	14.4/7.5 (1.9)
U. pugnax	13.5	15.6/6.0 (2.6)	12.0/6.1 (2.0)	9.1/3.8 (2.4)	10.3/4.0 (2.6)
U. pugilator	13.8	18.9/2.6 (7.3)	13.9/3.8 (3.7)	3.8/1.7 (2.2)	6.6/3.0 (2.2)
U. pugilator	14.7	25.2/4.5 (5.6)	13.6/5.8 (2.3)	6.3/3.3 (1.9)	7.9/3.6 (2.2)

CL = carapace length (mm); the "ratio" values, weight of the muscle of the major side divided by weight of the homologous contralateral muscle, are shown in parentheses below each weight value.

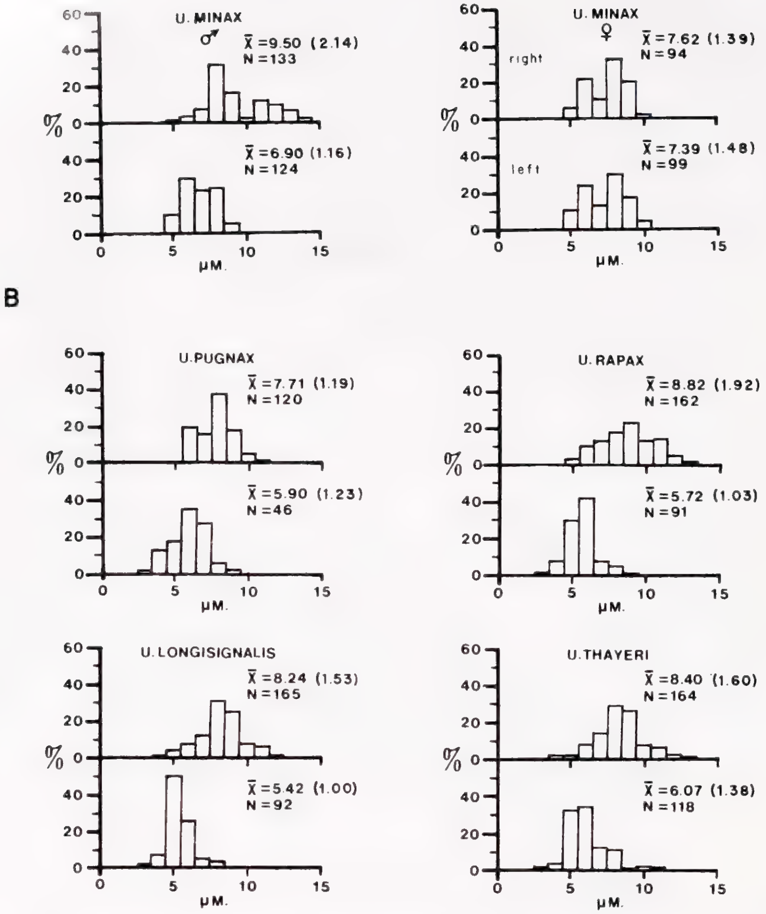

FIGURE 2. Comparisons of A-band lengths in major (upper figures) and minor (lower figures) levators of representative *Uca* of the subgenera *Minuca* and *Boboruca*. A: male *U. minax* (CL = 19.0 mm); female *U. minax* (CL = 20.2 mm). B: *U. pugnax* (CL = 12.0 mm), *U. longisignalis* (CL = 15.7 mm), *U. rapax* (CL = 13.5 mm) and *U. thayeri* (CL = 16.8 mm). $\bar{X}$ = mean A-band length (and SD) in μm from measurements of N fibers.

has a similar, less dramatic, asymmetry, with mean A-band lengths of 5.19 μm and 4.65 μm for the major and minor levator muscles (significant at 5% level).

Table II summarizes data from representative remotor muscles from seven species of *Uca*. Comparisons were made between homologous major and minor muscles of the same individual except in *U. pugilator* where it was necessary to compare muscles from two male crabs of similar size. These muscles of the major sides, including those of *U. pugilator*, had fiber populations with greater mean A-band lengths than those of the minor sides. All comparisons of means were highly significant ($P < 0.005$).

Data for both major and minor depressor and promotor muscles were taken for two species, *U. minax* and *U. pugilator*, and reflected similar asymmetry in fiber composition. For example, in a male *U. minax* (CL = 19.4 mm) means of A-band

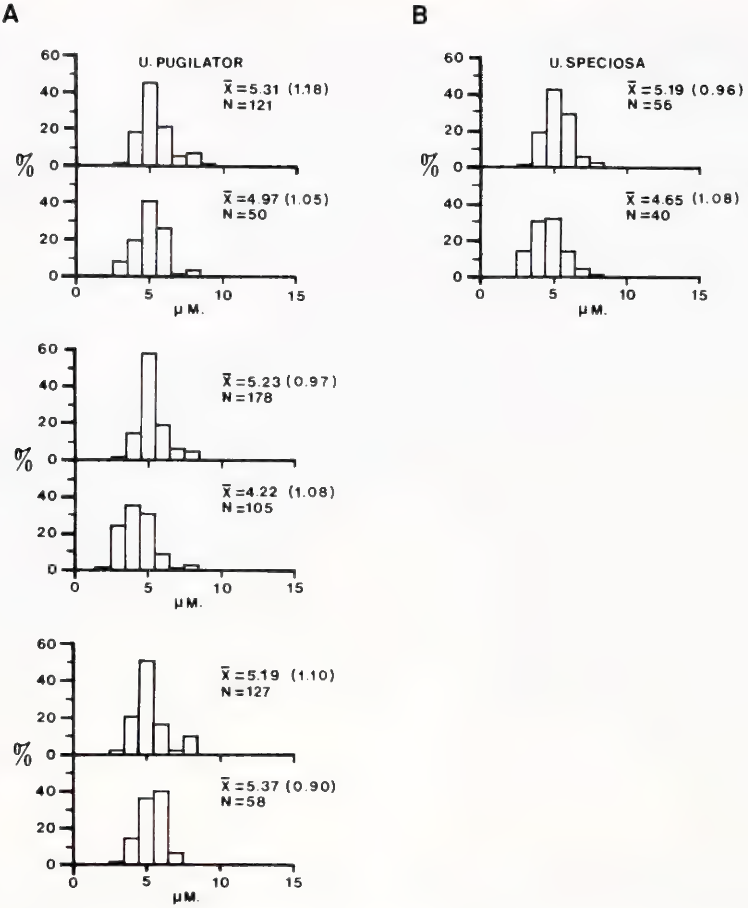

FIGURE 3. Comparisons of A-band lengths in major (upper figures) and minor (lower figures) levators of representative adult males of the subgenus *Celuca*. A: *Uca pugilator*, top to bottom, (CL = 16.6 mm, 16.9 mm, 14.8 mm). B: *U. speciosa*, (CL = 11.3 mm). X̄ = mean A-band length (and SD) in μm from measurements of N fibers.

lengths for major/minor promotors were 7.45 μm/6.42 μm, and for major/minor depressors were 8.99 μm/6.69 μm. Both comparisons were highly significant. Data for promotor and depressor muscles have not been taken for the other five species.

No asymmetry is evident in female fiddler crabs. Figure 2 gives A-band lengths from the two minor (right and left) levator muscles of an adult female *U. minax*. Means and distributions are very similar for both muscles; differences are not significant.

Muscles of the cheliped

The cheliped consists of seven segments: coxa, adjacent to the body; basis and ischium (fused); merus; carpus; propus; and dactyl. The moveable dactyl opposes the pollex, a distal projection of the propus, forming a claw or chela. The cheliped contains six major muscles; four of these, the extensor, stretcher, flexor, and bender, effect

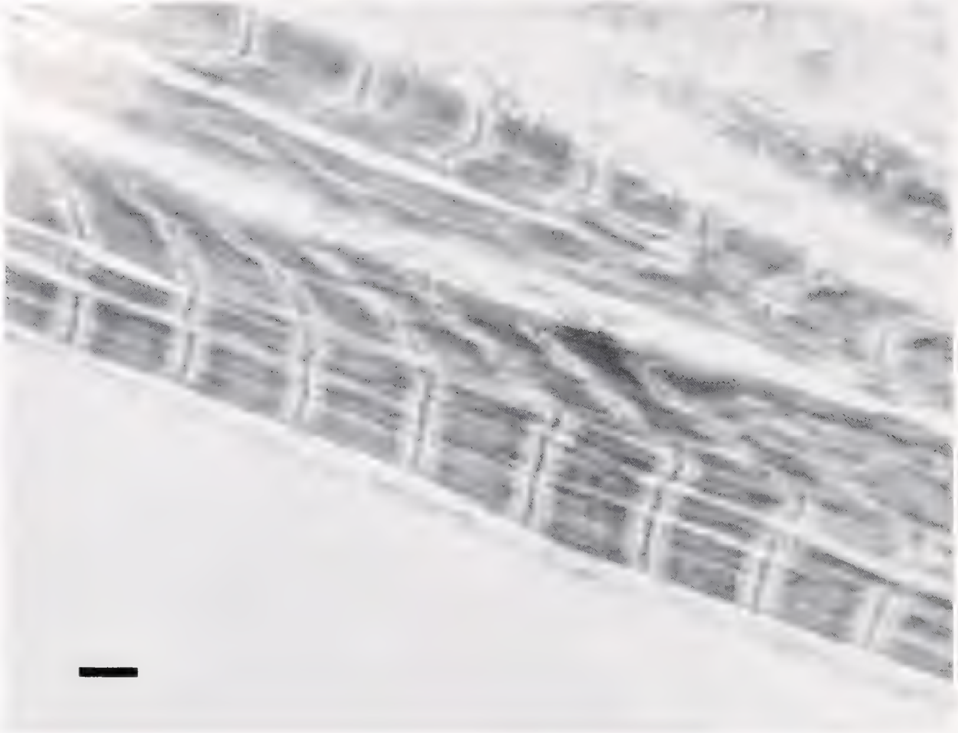

FIGURE 4. *Uca minax* major levator muscle fibers with 20–21 μm sarcomeres. FAA; 8 μm paraffin section; Mallory's triple stain; phase contrast. Scale bar = 10 μm.

extension and flexion of the limb during courtship waving. Contraction of the largest of the major chela muscles, the extensor located in the merus, causes extension of the chela at the merocarpopodite joint, a major element of the chela wave. The smaller flexor muscle, also located in the merus, is antagonistic in action to the extensor.

TABLE II

A-band measurements from fibers of remotor muscles from adult male fiddler crabs

		Major				Minor			
Species	CL	$\bar{X}$	SD	R	n	$\bar{X}$	SD	R	n
Uca minax	18.0	6.59	0.84	4–8	72	5.89	0.89	4–8	48
U. pugnax	12.0	6.92	1.31	5–11	67	5.70	1.15	5–11	43
U. rapax	13.5	7.46	1.15	5–11	92	6.24	0.92	4–9	53
U. longisignalis	15.7	6.61	0.90	4–9	78	5.32	0.93	3–7	62
U. thayeri	16.8	6.62	1.22	4–9	80	5.70	0.88	4–8	41
U. pugilator	15.9	7.40	0.85	6–9	63				
	14.3					6.45	1.00	5–8	58
U. speciosa	11.3	6.72	0.71	5–8	70	5.36	1.03	3–8	35

CL = carapace length in mm, n = number of fibers measured, major = hypertrophied cheliped, minor = other cheliped. Means ($\bar{X}$), standard deviations (SD) and ranges (R) in μm.

Contraction of the stretcher muscle in the carpus, causes extension of the chela at the carpopropodite joint, the bender muscle operates antagonistically to it. The muscles in the minor chela are similarly arranged, but there is a significant difference in its articulation. On the major side the contractions of the extensor and stretcher muscles cause the entire cheliped to be extended in a single plane, an essential component of the display wave of many species of *Uca*. On the minor side, the stretcher muscle causes a movement nearly perpendicular to that caused by the extensor muscle. Cheliped musculature in *U. pugnax* is illustrated and described in detail by Spirito (1970).

The weights and weight-ratios of cheliped muscles in two male *U. minax* and a male *U. pugilator* are given in Table III. In both species the extensors, stretchers, and benders of the major chelipeds are approximately 20 times larger than those of the minor contralateral chelipeds. Major flexor muscles of both species were approximately five times larger than their minor counterparts. In these animals the entire major chelipeds weigh 17–22 times the minor cheliped, and account for 32–47% of the entire body weight.

Figure 5 illustrates data from two male *U. minax*. The stretcher and bender muscles in the major carpus (upper half of each figure) have significantly greater mean A-band lengths than those of the minor carpus ($P < 0.05$). However, the extensor and flexor A-band lengths are indistinguishable between the major and minor chelae. For another *U. minax* (CL = 18.3 mm) data for both major and minor stretcher A-band lengths had means of 7.56 μm (SD 1.37, n = 38) and 5.41 μm (SD 0.84, n = 29) respectively; highly significant using student's *t*-test ($P < 0.001$). For a male *U. pugilator* (CL = 16.9 mm) mean A-band lengths in the major and minor flexor muscles were 6.52 μm (SD 0.82, n = 28) and 5.34 μm (SD 0.99, n = 51) respectively, again highly significant by student's *t* test ($P < 0.001$).

Variability of muscle fibers

Both A-band and sarcomere lengths were measured from the same fibers of the 15 thoracic and seven limb muscles which manipulate the chelipeds, of *U. minax*, *U. pugilator*, and *U. pugnax*. There was considerable variability in the ratio between these two length measurements, ranging from 0.46 to 0.68 (mean: 0.56) for thoracic muscle fibers and from 0.55 to 0.67 (mean: 0.59) for limb muscle fibers. These data are consistent with the suggestion that measurements of entire sarcomeres are more

TABLE III

Chela muscle weights (mg) from three Uca *(same individuals as in Table I)*

		Major/minor chela muscle weights (major/minor ratio)			
Species	CL	Extensor	Flexor	Stretcher	Bender
U. minax	20.7	228.0/16.5 (13.8)	47.1/8.0 (5.9)	112.0/5.0 (22.4)	49.6/3.6 (13.8)
U. minax	19.9	156.0/11.1 (14.1)	36.9/9.0 (4.1)	72.6/3.9 (18.6)	34.6/3.1 (11.2)
U. pugilator	13.8	58.5/6.2 (9.4)	10.0/3.7 (2.7)	34.0/1.4 (24.3)	15.1/0.8 (18.9)

CL = carapace length (mm); the "ratio" values, weight of the specified major chela muscle divided by weight of the homologous minor chela muscle, are shown in parentheses below each weight value.

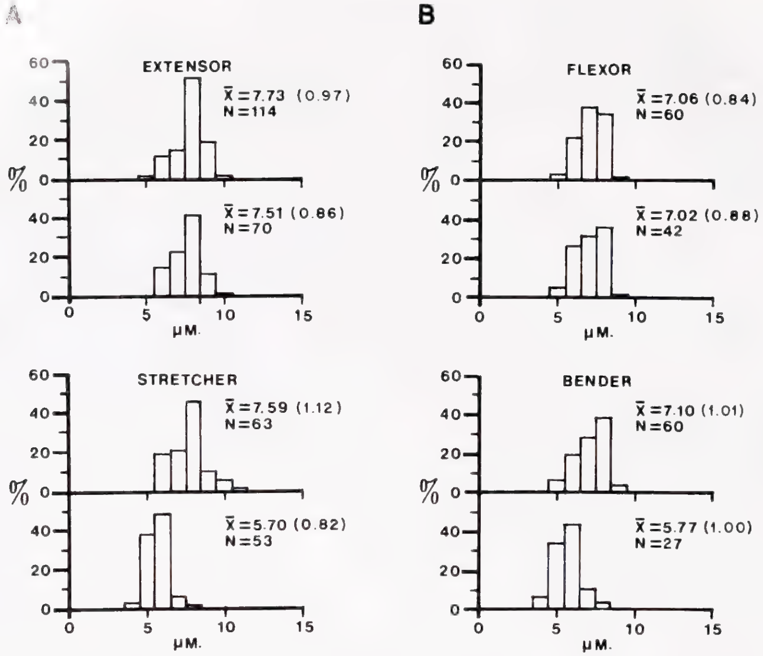

FIGURE 5. Comparisons of A-bands lengths in major (upper figures) and minor (lower figures) chela muscles from two adult male *U. minax*. A: (CL = 19.4 mm), extensor and stretcher; B: (CL = 19.9 mm), stretcher and bender. $\bar{X}$ = mean A-band (and SD) in μm, from measurements of N fibers. For major extensors and stretchers only 20% of all fibers were measured.

prone to artifactual variability due to fiber stretch during fixation (Franzini-Armstrong, 1970), the thoracic muscles being more difficult to position for fixation. For this reason only A-band measurements are used elsewhere in this report.

To estimate variation in length of A-bands from single fibers, multiple A-band measurements were made along the longitudinal axes of 12 representative fibers from levator muscles of four specimens of three species of *Uca* (Table IV). At a given point on a fiber, variation in A-band length was insignificant in the direction of the short axis, but along the longitudinal axis variation was substantial, with longer A-bands at the ends of the fibers. The Coefficient of Variation (CV) of each fiber is not related to mean A-band length for different muscle fibers from the same individual. The data presented (Table IV) show substantial variability within fibers, with CV values ranging from 7.0 to 18.8. In other data (Rhodes, 1977) there is even greater variability *between* fibers of a muscle. However CV values show no discernable trends between muscle A-bands of the major or minor sides, or between species. For example, in a comparison of the major levators of four large *U. minax* (mean CL: 20.1 mm) and four *U. pugilator* (mean CL: 16.1 mm), mean A-band lengths were 8.92 μm and 5.49 μm respectively. CV values for these means were 22.3 and 20.6.

Isometric tension

In preliminary comparisons of rates of isometric tension development in major levators of *U. minax* and *U. pugilator*, the major levator muscles of *U. minax* show slow, non-facilitating tension development to stimulus frequencies of 1 Hz. At 3 Hz

TABLE IV

Variation in A-band length (μm) for single fibers of levator muscles of four Uca *of three species*

Species	Side	Fiber #	Sample	$\bar{X}$	CV	Range
U. longisignalis	major[1]	1	13	11.11	16.2	8.7–13.3
		2	15	10.97	18.8	7.2–14.1
U. minax	major[1]	1	34	7.96	11.1	6.1–9.9
		2	27	10.71	13.6	8.0–12.5
		3	15	9.88	16.2	6.1–12.2
		4	18	9.16	15.9	6.1–11.4
	minor	1	28	8.74	12.9	7.2–10.6
		2	52	5.21	18.0	3.0–6.8
		3	31	7.82	15.0	6.1–11.4
U. pugilator	major[1]	1	33	4.87	9.0	3.8–5.7
		2	19	5.30	7.0	4.6–5.7
		3	20	5.04	7.3	4.2–5.7

[1] The posterior branch of the major levator muscle was not sampled.
CV = coefficient of variation, $\bar{X}$ = mean A-band length.

the individual responses begin to fuse. At stimulus frequencies of 10 Hz and higher the muscle responds tetanically and continues to develop tension even after the stimulus is removed. In one instance a levator of *U. minax* developed a tetanic tension equivalent to 100 g, whereas levator muscles of a similar size from *U. pugilator* rarely develop tension in excess of 10 g. These latter muscles exhibit distinct twitch-like contractions at stimulus frequencies of 10–15 Hz. These responses of whole muscles are consistent with what would be predicted from their muscle fiber composition (Atwood, 1972, 1973; Josephson, 1975).

Correlation of A-band length and wave duration

Waving in fiddler crabs is species-specific in terms of the pattern, duration, frequency, smoothness, etc. (Schöne, 1961; Crane, 1975). In order to determine whether differences in waving are due to neuromuscular properties, A-band lengths were correlated with waving behavior. Since the levators, extensors and stretchers are involved in the extension and elevation of the major claw, one might expect slow wavers to have functionally slow muscles (long A-band lengths) and fast wavers to have functionally fast muscles (short A-band lengths) (del Castillo *et al.*, 1972; Josephson, 1975). No correlation should be seen between A-band lengths of the remotor muscle and wave duration since the actions of this muscle (*i.e.*, the backward movements of the claw during the wave) are aided by gravity when the front of the animal is held up by the walking legs.

Table V lists correlations between the estimated wave durations of six species of *Uca* (Crane, 1975; Salmon, 1965, 1967; Salmon and Atsaides, 1968b; Doherty, 1982) and the grand means of all A-band measurements from four muscles from the same species (Rhodes, 1977). The wave duration values are not very satisfactory; as Doherty (1982) has shown, there is a marked negative correlation between wave duration and temperature for at least two of these species—a correlation which was not considered in earlier studies. Despite this problem, the comparisons of rank are significant ($P < 0.05$) for the levator and extensor muscles. The Spearman rank correlation tests are not significant at this level for the stretcher muscle; the remotor muscle, as predicted,

TABLE V

Comparisons of moderate intensity wave durations and means of all A-band lengths for six species of Uca

	Duration				Muscle							
					Levator		Remotor		Extensor		Stretcher	
Species	Salmon[1]	Crane[2]	Doherty[3]	Rank	μm	Rank	μm	Rank	μm	Rank	μm	Rank
U. speciosa	0.50	0.38–0.5		1	4.75	1	5.89	1	5.85	1	6.21	2
U. pugilator	1.25	0.79–1.8		2	5.49	2	7.40	5	6.23	2	6.10	1
U. minax	2.5[4]	2–3[4]	4–5	3	8.92	4	6.89	4	7.57	4	7.12	4
U. pugnax	2.1–3.7	0.87–5	9–10	4	7.47	3	6.52	2	7.63	5	7.19	5
U. rapax	3.6	2.1–12		5	9.16	6	7.50	6	7.17	3	7.11	3
U. longisignalis	5.6			6	9.03	5	6.61	3	7.76	6	7.53	6
					r_s = .886 sig.		r_s = .314 n.s.		r_s = .829 sig.		r_s = .771 n.s.	

[1] Salmon (1965, 1967); Salmon and Atsaides (1968b).

[2] Crane (1975).

[3] Doherty (1982) at 25°C.

[4] Waves in response to a female. Both authors suggest longer duration for moderate intensity waves.

n.s. = not significant; r_s = Spearman rank coefficient; sig. = significant at 5% level.

shows no correlation (Table V). These results indicate that the functional properties of the peripheral neuromuscular system correlated with sarcomere length may constrain the spatio-temporal components of the wave display and thus play a role in shaping the evolution of this species-isolating mechanism.

Discussion

The major internal modifications of the skeleton, muscles, and nervous system necessary to accommodate the great chela of male *Uca* include an asymmetry of the thoracic skeleton, a marked asymmetry in the thoracic ganglionic mass of the central nervous system (Huxley, 1932; Rhodes, 1977; Young and Govind, 1983), significant differences in the cross-sectional area of certain large (presumably motor) axons innervating the major chela muscles (Rhodes, 1977; Young and Govind, 1983), as well as the major muscle asymmetries reported here. The thoracic musculature of *Uca* with its associated skeleton (Fig. 1) reflects the gross asymmetry of the two chelae in male crabs. The major body muscles are three-fold or more greater in weight than those of the minor side, or of the homologous muscles of females of the same size. Hypertrophied muscles within the distal parts of the chela may be 20 times larger than their minor homologues.

In the more primitive species (Crane, 1975) muscle fiber lengths increase with body size but are not significantly different between major and minor sides of individual animals, even when the major muscle is hypertrophied. Sarcomere lengths, as measured by A-band lengths, are significantly larger in the thoracic muscles that manipulate the major chelipeds, compared to their minor homologues. Since the major chela is huge relative to the rest of the body, such modifications seem appropriate to the specialized role of these muscles. Waving movements by the male fiddler crab of his major cheliped must require muscles capable of developing more tension than that required to move the relatively small minor cheliped during feeding and burrowing. A portion of this power is furnished by an increase in the size of the thoracic muscles of the major side; however, the extent of such evolutionary hypertrophy may be limited by constraints imposed by the exoskeleton. Another way that the power of these muscles could be increased is by modification of the contractile machinary. Increased sarcomere length provides for greater overlap of actin and myosin filaments, the formation of more actomyosin bonds and hence of greater tension development by individual muscle fibers. Thus increasing both the size and the sarcomere lengths of the muscles that manipulate the major chela seem appropriate evolutionary responses for fiddler crabs. In several species, and particularly in the largest species studied, *U. minax,* major levator muscles are characterized by fibers with sarcomeres in excess of 20 μm. Previously reported long sarcomeres of skeletal muscle have been shown to be extremely slow and powerful (del Castillo *et al.,* 1972; Atwood, 1973; Josephson, 1975). If these functional correlates hold true for the thoracic muscles of fiddler crabs, then on a weight-specific basis these muscles are unusually powerful but very slow as well.

In contrast to the situation in most species of *Uca,* the hypertrophied major levator muscles of *U. pugilator* and *U. speciosa* show little or no difference in A-band lengths compared to their contralateral minor levator muscles. These muscles are relatively large compared to the major levator of *Minuca* species and the fibers are also relatively long. Increased muscle size without elongated sarcomeres thus represents a substantial evolutionary specialization by members of the *Celuca* sub-genus, to allow manipulation of the large cheliped without sacrificing speed, permitting faster waves and sound production. Sound production by vertical drumming of the flexed major cheliped at frequencies of 6–10 Hz (Salmon and Atsaides, 1968b; Salmon *et al.,* 1978) is characteristic of the *Celuca* subgenus, but not of the subgenus *Minuca* (see Crane, 1975).

The latter species raise and lower the flexed major cheliped at no more than 1 Hz when courting. Thus it appears that the functionally slow but powerful thoracic muscles [illegible] and other primitive *Uca* preclude the ability to drum at a frequency that would produce sound.

These findings from species of *Uca* are supportive of the hypothesis that motor patterns generated in the central nervous system are very conservative evolutionarily, so that species-isolating behaviors are affected by more malleable peripheral elements, including the morphological and physiological properties of the muscle fibers and their associated motor axons. As Hoyle (1976) has pointed out, in supporting this possibility, "Why, otherwise, would there be such diversity in the muscles?" In the case of *Uca* the degree of muscle dimorphism could determine the duration and frequency of the courtship wave. The capacity for sound production in *Celuca* species could be determined by the type of muscle hypertrophy they have evolved to manipulate their huge major chela.

ACKNOWLEDGMENTS

Figure 1 was drawn by Ms. Cheryl Hughes. Editorial assistance in the revision of this paper by J. A Doherty, L. M. Passano, V. E. Trinkaus-Randall, and by several anonymous reviewers is gratefully acknowledged. This research was funded by NSF Grant #BNS 75-19641 to Dr. L. M. Passano and by grants from the Graduate School, University of Wisconsin-Madison.

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EFFECT OF Y-ORGAN ABLATION ON OOCYTE GROWTH IN THE TERRESTRIAL ISOPOD, *ARMADILLIDIUM VULGARE*

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ABSTRACT

The reproductive and molting cycles of the isopod, *Armadillidium vulgare,* are synchronous during its breeding season. It was found that the rapid growth of oocytes occurs at stage D of the molting cycle. In hibernating females, the oocytes ceased to grow if their Y-organs were surgically extirpated at stage C in early spring. In these animals, many oocytes devoid of follicle cells degenerated along the inner margin of the ovary. Occasionally, however, a mass of granular substance was also seen in that part of the ovary. These results suggest that in *A. vulgare* the rapid growth of oocytes in stage D females is closely associated with activity of the Y-organ.

INTRODUCTION

In the terrestrial isopod *Armadillidium vulgare* as in many other species of Crustacea (Meusy and Charniaux-Cotton, 1984; Charniaux-Cotton, 1985), the reproductive and molting cycles are synchronous (Nakatsuchi, 1983). This suggests that the Y-organ, which is responsible for production of the molting hormone, may influence reproduction.

Cauterization of the Y-organs of the amphipod *Orchestia gammarella* inhibited further growth of oocytes and vitellogenin synthesis (Meusy *et al.,* 1977; Mathieu-Capderou, 1980; Blanchet-Tournier, 1982). In the isopods *Idotea balthica* (Reidenbach, 1971) and *Porcellio dilatatus* (Souty *et al.,* 1982), as well, cauterization of Y-organs resulted in arrested oocyte growth and lowered the rate of vitellogen release into the hemolymph. This method of cauterization of the Y-organs may produce some degenerated substances, which in turn exert an unfavorable influence upon the animal. A more desirable method is to ablate Y-organs by surgical extirpation.

The present study was undertaken to ascertain if surgical extirpation of the Y-organs arrests the growth of oocytes in *Armadillidium.* As observed under a dissecting microscope, the ovary of this species is a paired organ which extends longitudinally on both sides of the animal's cavity and is of modest size.

MATERIALS AND METHODS

Animals

Specimens of terrestrial isopod, *Armadillidium vulgare,* used in this investigation were collected in February and March around Yokohama when the field population was in hibernation. Only adult females, 10–12 mm in body length, were used. Collected females were divided into the following five groups: initial specimens (Group A), animals with sham-operated Y-organs (Groups B and C), and animals with ablated

Received 29 October 1985; accepted 16 January 1986.

Y-organs (Groups D and E). After operation, several females were kept in each petri dish with two adult males. Males and females were together for mating and oviposition by the females. The dishes containing moistened soil were placed in the laboratory at 25 ± 2°C and in natural daylight. Animals were fed decayed leaves and rat chows. Operations and observations were performed under a dissecting microscope. Surgical ablation of Y-organs was performed within a few days following collection. Animals were dissected after 25 (Groups B and D) and 50 (Groups C and E) post-operative days. Control animals (Group A) were similarly killed on the day the experiment started. The oocyte diameter was measured in ten largest oocytes of fresh ovaries in each animal.

To examine the interrelation between normal oocyte growth and the molting cycle, about fifty females were similarly selected and reared. They were sacrificed at three day intervals for observations on the development of the ovaries. Their fresh ovaries were observed with a light microscope.

The paraffin-imbedded ovaries of some females were sectioned 10 μm thick and stained with Mayer's hematoxylin and eosin.

Surgical ablation of Y-organs

The Y-organs of *A. vulgare* are bilaterally located at the base of the maxillary segment (Figs. 1A–D). The molt-controlling function of this organ has already been demonstrated by extirpation and reimplantation experiments (Suzuki, 1983).

A few days after collection, Y-organs were pulled from the females through a small hole using a pair of fine forceps. Operations were performed without any other treatment to the wounds. In a sham-operated animal, Y-organs were pulled out with forceps but then immediately placed back on their original sites. Success of the operation of the Y-organ ablation was ascertained by histologically studying the animals at the end of the experiment. As a result, it was verified that the Y-organs had been perfectly extirpated in all animals of Groups D and E.

RESULTS

Observation concerning normal oocyte growth

The collected animals were at stage C of the molting cycle, had no sternolith on the thoracic segments (Madhavan, 1981), and had a pair of ovaries containing about one hundred oocytes (50–200 μm diameter) in each ovary (Fig. 2A and Group A in Table I). Larger oocytes (opaque yellow in fresh ovary) were located along the inner margin of the ovaries. Gradually the oocytes grew and were surrounded by a single layer of follicle cells. The sternoliths appeared about two weeks after collection, marking the beginning of stage D of the molting cycle. At that time most oocytes were approximately the same size (about 300 μm diameter). Rapid oocyte growth occurred in animals at stage D. One week later all oocytes had grown synchronously to become 500 μm in diameter, and were bright yellow (Fig. 2B). Ecdysis occurred first in the posterior region of the body, and one day later in the anterior region. Females laid about 80 eggs (530–650 μm diameter) in a pouch formed by the oostegites within 24 h after the anterior ecdysis. Forty-eight hours after the first oviposition, young oocytes (50–100 μm diameter) were seen lined up along the outer margin of the ovary (Fig. 2C). The young oocytes gradually increased in size and moved toward the inner margin of the ovary. Laid eggs were kept in the oostegites of ovigerous females for 16–19 days before larvae were released. About a week after release of the larvae, the remaining young oocytes grew rapidly again for the next oviposition soon after the appearance

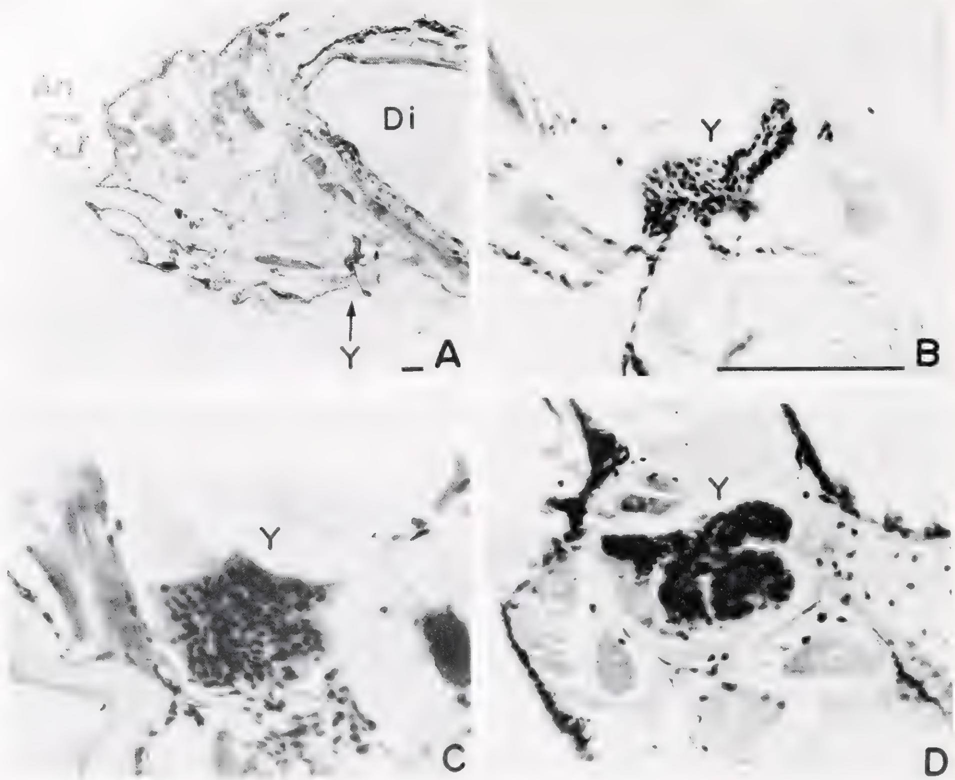

FIGURE 1. Location of Y-organs in vertical (A and B), horizontal (C) and transverse (D) sections of the head of *Armadillidium vulgare.* B is an enlargement of A through the Y-organ. Paraffin sections stained with hematoxylin-eosin. An, antenna; Di, digestive organ; Y, Y-organ. Scale bar = 100 μm.

of the sternoliths. During the experiment, almost all females laid the first and second eggs of the year in succession after each ecdysis.

Effect of Y-organ ablation on oocyte growth

Control females with Y-organs molted once (Groups B and C) or twice (Group C) during the experimental period (Table I), with their oocytes increasing in size. On the 25th day, about half the animals of Group B deposited eggs after molting and the other animals had large oocytes in their ovaries (Fig. 2D). On the 50th day, large oocytes for the secondary oviposition were also detected in the ovaries of Group C females. Some of them (30%) laid eggs in succession after the next molting.

No appreciable increase in oocyte diameter was noted during the experimental period in the Y-organ ectomized females (Groups D and E in Table I). The mean oocyte size in animals of Groups D and E was almost the same as in Group A, showing no increase in oocyte size in these animals. Moreover, exuviation and oviposition were completely blocked. On the 25th day following Y-organ ablation, most animals of Group D contained many oocytes, irregular in shape, along the inner margin of the ovary (Fig. 2E). On the 50th day, there were many traces of degenerating oocytes

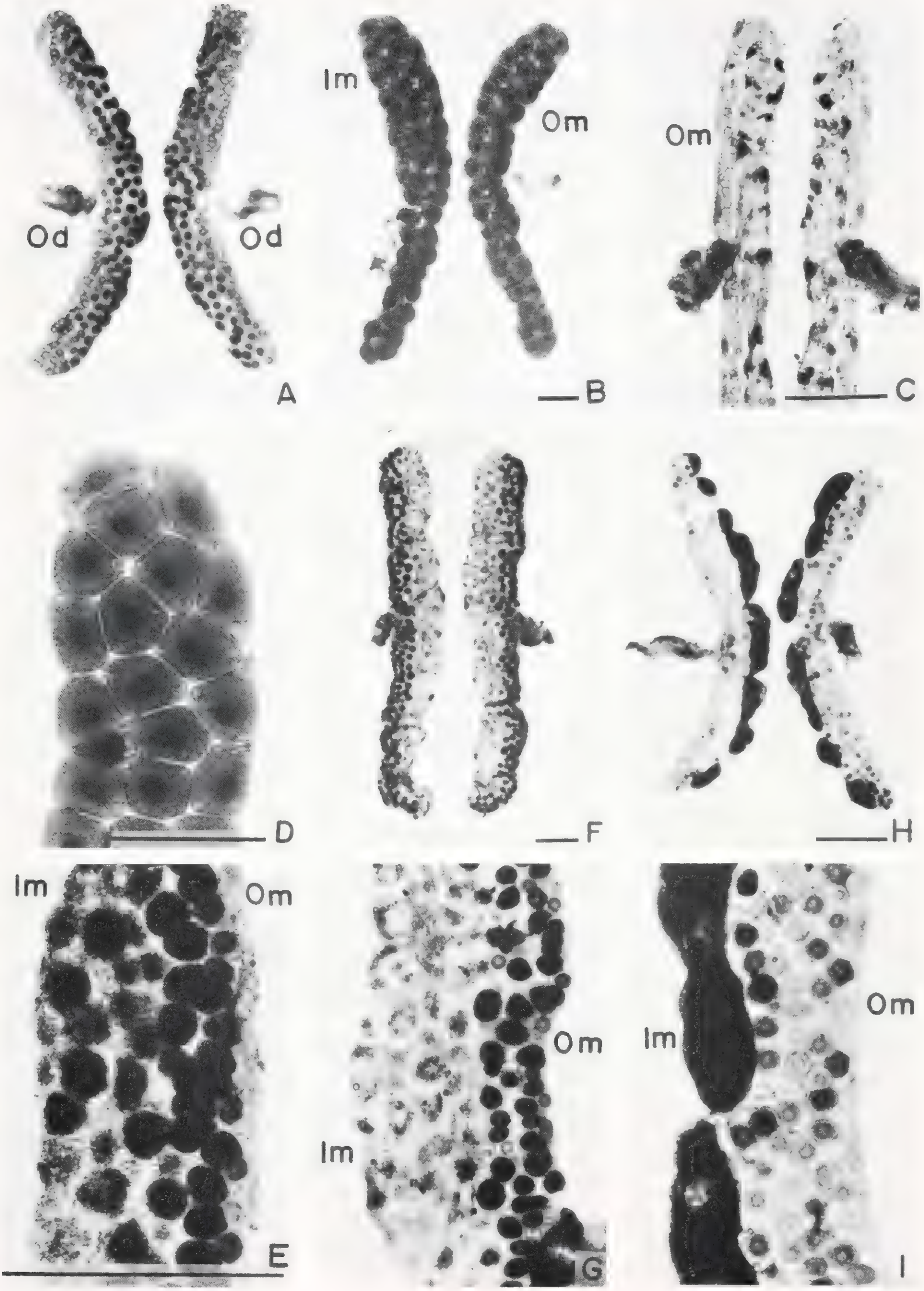

FIGURE 2. Photomicrographs of fresh ovaries of normal (A–D) and Y-organ ablated (E–I) females. A is 0 day, B, D, and E are 25 days, and F–I are 50 days after the beginning of the experiment. C represents the second day after oviposition. G and I are enlargements of F and H, respectively. Od, oviduct; Im, inner margin of the ovary; Om, outer margin of the ovary. Scale bar = 1 mm.

TABLE I

[illegible] gan ablation on oocyte growth of Armadillidium vulgare

Groups	Days after operation	No. of females	Oocyte diameter μm, mean ± S.D.	Percent of molted females	Percent of egg-laid females
A Initial specimens	0	15	184.8 ± 31.5	0	0
B Sham-operated	25	15	497.1 ± 125.5*	80.0	53.3
C Sham-operated	50	9	587.2 ± 31.2* 349.1 ± 203.2**	100	100
D Y-organs ablated	25	14	232.7 ± 31.6	0	0
E Y-organs ablated	50	12	203.5 ± 41.2	0	0

The oocyte diameter shows the mean size of ten largest oocytes of each animal at the time of experiment termination. When animal laid eggs during the experiment, it shows the mean diameter of ten laid eggs for the first (*) and second (**) oviposition.

in the inner margin of the ovary of many Group E animals (Figs. 2F, G) and follicle cells could not be observed around these degenerating oocytes. On the other hand, in some females of Group E (about 20%) a mass of granular substance (opaque yellow in fresh ovary and eosin positive) was observed along the inner margin of their ovaries (Figs. 2H, I). These ovaries (Fig. 2I) did not have as many degenerating oocytes as others of Group E (Fig. 2G). However, in the outer margin of the ovary of all Group D and E animals there were young and medium sized oocytes (Figs. 2E, G, I), with as normal an appearance as those of control females with Y-organs (Groups B and C).

DISCUSSION

In early spring *Armadillidium vulgare* females are in stage C of the molting cycle and have only young and medium size oocytes in their ovaries. The oocytes grow gradually for a while; rapid growth occurs abruptly when animals reach stage D. In this species, oocyte growth is coupled with the molting cycle; Nakatsuchi (1983) has already reported that the female reproductive cycle (vitellogenesis and oviposition) of this species is coupled with its molting cycle.

The present study shows that in the terrestrial isopod, *A. vulgare,* the presence of the Y-organ is needed for oocyte growth. The same result was already obtained in other isopods and amphipods as stated in the Introduction. The effect on isopod ovaries would be no different if the Y-organs were surgically extirpated or cauterized.

If surgical extirpation of the Y-organs was performed on *A. vulgare* at stage C, follicle cells would degenerate and oocytes would also degenerate in many animals (Figs. 2E, G). However, two different ovarian reactions were observed on the effect of Y-organ ablation, as shown in Figures 2G and I. These different reactions are left for further study.

Oviposition does not take place at every exuviation in *Armadillidium,* as shown in Table I (Group B). Other factors may exist for oocyte growth besides the molting hormone (Y-organ). In the female spider crab, *Libinia emarginata,* ovarian development occurs after the degeneration of the Y-organs (Hinsch, 1972). It is also postulated that a protocerebral factor is involved in the control of oocyte growth of the amphipod (Blanchet-Tournier *et al.,* 1980; Blanchet-Tournier, 1982). In the isopod, however, the protocerebrum appears to release a gonad-inhibiting hormone (Legrand *et al.,* 1982). It is possible that different Crustacea have developed various modes of hormonal control of the reproductive cycle (Meusy and Charniaux-Cotton, 1984; Charniaux-Cotton, 1985).

ACKNOWLEDGMENT

The author wishes to thank to Prof. Y. Katakura of Keio University for his helpful suggestions during the course of this work.

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PRINCE & LEMON STS.
LANCASTER, PA

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Woods Hole, Mass.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $20.00. Subscription per volume (three issues), $50.00 ($100.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor, or Pamela Clapp, Assistant Editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

POSTMASTER: Send address changes to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory,
Woods Hole, MA 02543.
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ISSN 0006-3185

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JUNE, 1986

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

The BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $20.00. Subscription per volume (three issues), $50.00 ($100.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor, or Pamela Clapp, Assistant Editor, Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

THE BIOLOGICAL BULLETIN (ISSN 0006-3185)

POSTMASTER: Send address changes to THE BIOLOGICAL BULLETIN,
Marine Biological Laboratory, Woods Hole, MA 02543.
Second-class postage paid at Woods Hole, MA, and additional mailing offices.

LANCASTER PRESS, INC., LANCASTER, PA

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CHEMOATTRACTION IN *TETRAHYMENA*: ON THE ROLE OF CHEMOKINESIS

PER HELLUNG-LARSEN†, VAGN LEICK†, AND NIELS TOMMERUP*

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ABSTRACT

Chemoattraction of *Tetrahymena pyriformis,* strain GL, was measured during starvation and under different growth conditions. Log phase cells starved in buffer are attracted by certain amino acids, peptides, and proteins. Cysteine, methionine, and phenylalanine are attractants at 10^{-4} M. The peptides in proteose peptone (PP) and yeast extract (YE) are active at 10^{-6} M. Epidermal growth factor (EGF) is active at $>3 \times 10^{-5}$ M. Among the proteins, platelet derived growth factor (PDGF) is the most active (3×10^{-8} M). Cells growing in defined medium are attracted by PP, YE, and some proteins (PDGF).

Swimming speed was measured for starved cells with and without added attractants or repellents. With addition of PP the swimming speed increases from 0.42 to 0.51 mm/s., but for PDGF it is unchanged. The swimming speed of starved cells increases when the cells approach a solidified attractant (PP) as measured by the speed at a given distance. The speed of cells moving towards the attractant is higher than that of cells moving away from it.

In conclusion, certain amino acids, peptides, and proteins are chemoattractants for *Tetrahymena.* Chemokinesis likely plays a considerable role in the case of PP (and YE), since they increase swimming speeds, whereas attraction by PDGF may involve chemotaxis.

INTRODUCTION

Detailed studies on the chemosensory response of ciliated protozoa have been performed with *Paramecium* (Dryl, 1973; Van Houten *et al.,* 1982) and with *Tetrahymena* (Almagor *et al.,* 1981; Levandowsky *et al.,* 1984).

The chemosensory response can be considered as chemotaxis and/or chemokinesis. Chemotaxis refers to an oriented movement—the cell orients itself and moves towards or away from the chemical source—whereas chemokinesis denotes an unoriented change of swimming speed (orthokinesis) or turning/tumbling frequency (klinokinesis) by unoriented cells which accordingly use the gradually increasing speed to reach the attractant (Lapidus and Levandowsky, 1981).

Recently we described the effect of Platelet Derived Growth Factor (PDGF) on chemoattraction of *Tetrahymena* (Andersen *et al.,* 1984). Cells starved in buffer are also attracted by amino acids and peptides whereas cells taken directly from Holz's defined medium (Holz, 1964) as modified by Rasmussen and Modeweg-Hansen (1973)

Received 15 October 1985; accepted 25 March 1986.

Abbreviations: PP: Proteose peptone, YE: Yeast extract, PY-medium: Proteose peptone (0.75%), Yeast extract (0.75%), glucose (1.5%), $MgSO_4$ (1 mM), $CaCl_2$ (50 mM) and Ferric citrate (100 μM), DM: Defined Medium of Holz (1964) as modified by Rasmussen and Modeweg-Hansen (1973), IBMX: Isobutyl-methylxanthine, EGF: Epidermal growth factor, PDGF: Platelet derived growth factor, db-cAMP: dibuturyl-cyclic AMP.

responses towards the peptides in Proteose Peptone (PP) and Yeast Extract (YE) and towards PDGF (Leick and Hellung-Larsen, 1985).

In the present paper we have: (1) reinvestigated the chemoattraction of a number of components including the growth factors EGF and PDGF by using four different assays for registration of the cell accumulation, (2) correlated the chemosensory response to the swimming speed and to the intracellular cAMP level, and (3) measured the cell number at certain distances from a gelified attractant and measured the swimming speed towards and away from the attractant.

We conclude that the chemosensory response of *Tetrahymena* towards peptides, at least to a large extent, can be explained by a chemokinesis, whereas the response towards PDGF, which has no effect on the swimming speed or tumbling/turning frequency, may involve a taxis mechanism.

MATERIALS AND METHODS

Isobutyl-methyl-xanthine (IBMX) was purchased from Aldrich-Chemie, W. Germany. The serum substitute Ultroser G was obtained from LKB, Bromma, Sweden. Proteose peptone (PP) and yeast extract (YE) were purchased from Difco. The binding protein for the cAMP assay was kindly supplied by Dr. P. Thams and Dr. C. J. Hedeskov, Department of Biochemistry A, University of Copenhagen. The ^{3}H-labeled cAMP was purchased from Amersham (TRK 498).

Tetrahymena pyriformis strain GL was grown at 28°C in PY-medium (0.75% PP, 0.75% YE, 1.5% glucose, 1 mM $MgSO_4$, 50 μM $CaCl_2$, and 100 μM ferric citrate) or in a defined medium (DM) containing amino acids, ribosides, vitamins, and salts (Holz, 1964). Starvation of the cells was performed by centrifugation at 500 × *g*, a careful wash with 10 mM Tris-HCl, pH = 7.4, and resuspension in this buffer to a cell concentration of 10^5 cells/ml. The cells were starved for 16 h at 28°C.

The chemosensory response was studied at 25°C by four different methods: (1) *Capillary assay*—by use of an apparatus with horizontal capillaries for migration of the cells into a trap, which allows electronic counting of the accumulated cells (Leick and Helle, 1983). In most of the experiments reported here the outer chamber had a volume of 10 ml instead of 2 ml. The experiments were run for 2, 5, or 21 h. For further details see Leick and Helle (1983). (2) *Gel assay*—a qualitative assay for testing if substances gelified with 1% agarose (iself inert) were attractants or repellents. A small piece of gel ⅛–1 mm^3 was placed on a glass slide. Starved cells (10^4–10^5 cells/ml) were added and their distribution followed in a stereo microscope. (3) *Two-phase assay*—a semi-quantitative micro-assay with high sensitivity. Cells suspended in PY, DM, or Tris (10^5/ml) (25°C) were layered carefully in glass tubes on top of PY, DM, or Tris containing 5% of the radio opaque drug metrizamide and a defined concentration of attractant/repellent (Fig. 1). In the negative controls all cells stay in the upper phase, whereas the cells migrate into the bottom layer within 10 min if an attractant is present. If the cells are placed in the bottom layer with metrizamide they move towards the upper phase, *i.e.*, they try to avoid the metrizamide, which also slightly reduces their swimming speed. After addition of attractant the cells move downwards towards the attractant although they then have to approach the metrizamide. Gelatine, ficoll, and sucrose did not work either because they are attractants (gelatine) or because the cells get partially trapped in the bottom phase (ficoll and sucrose). (4) *Field-assay.* Analyses of cell accumulation and of swimming speed at different distances from gelified attractants/repellents: pieces of gelified attractants/repellents (⅛ mm^3) were placed at the edge of a glass slide to ensure access of oxygen to the cells when mounted with a coverslip. The object was placed on an automatic scanning stage on a Leitz

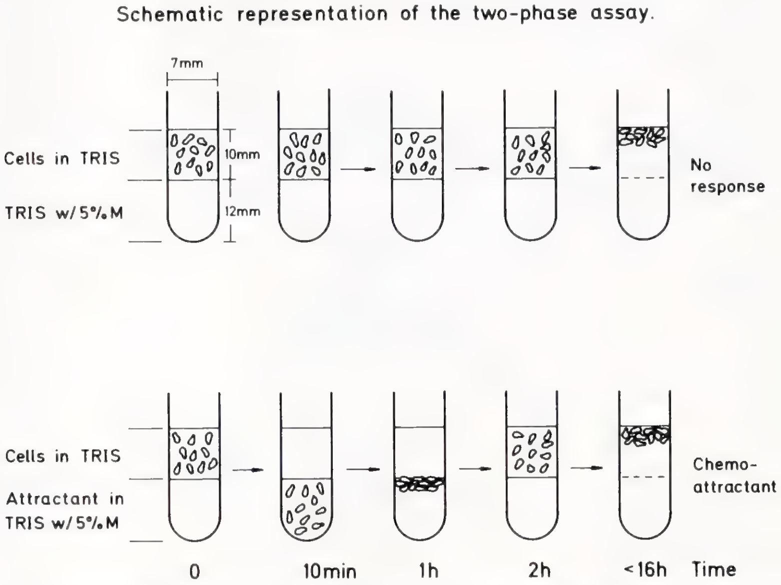

FIGURE 1. Schematic representation of the principle in the two-phase assay for chemoattraction. 400 μl *Tetrahymena pyriformis* log cells grown in PY, DM, or starved in Tris were adjusted to 10^5/ml at 25°C and carefully layered in glass tubes (inner diameter: 7 mm) on top of 400 μl PY, DM, or Tris containing 5% w/v metrizamide. The bottom phase was added attractant and/or repellent in the experimental tubes. Visual readings were performed at different times after set-up. In some cases the assay was performed in polyvinyl micro-cuvettes and the optical density at 450 nm of the bottom phase was registered after 10 minutes.

Periplan microscope coupled to a Leitz Tass Image Analyzer (objective 2½×, final magnification 7×). A program was developed to automatically count the cell density per field in 10 adjacent fields each measuring 2.4 × 2.1 mm. Whenever the last field had been analyzed the scanning stage automatically relocated the first field in focus and the analyses were repeated. Another program, which could be activated whenever desired, was developed to measure swimming speed and turning/tumbling frequency. Upon activation six successive images of the same field, each detected with a 200 ms delay, were fused within an image memory and shown on the video screen. Thus the track of each cell in an one-second period was directly visible and the swimming velocities were automatically calculated from the length of each track. Furthermore, turning/tumbling of a cell was directly visualized by deviation from a linear track (Fig. 2).

Intracellular levels of cyclic AMP were determined by a protein binding assay essentially as described by Geisler *et al.* (1977). Labeled cAMP and unlabeled cAMP (to be measured) compete for binding to a binding protein isolated from rabbit muscle. 2,8-^{3}H-Adenosine-3′,5′-cyclic phosphate ammonium salt (30–50 Ci/mmol) was diluted to 1 pmol/50 μl with assay buffer before use. Standards containing 0.25–12 pmol/50 μl of unlabeled cAMP were prepared from a stock solution.

RESULTS

The swimming behavior (swimming speed and turning/tumbling frequency) was studied in cells grown in PY-medium and starved in Tris-buffer and with starved cells

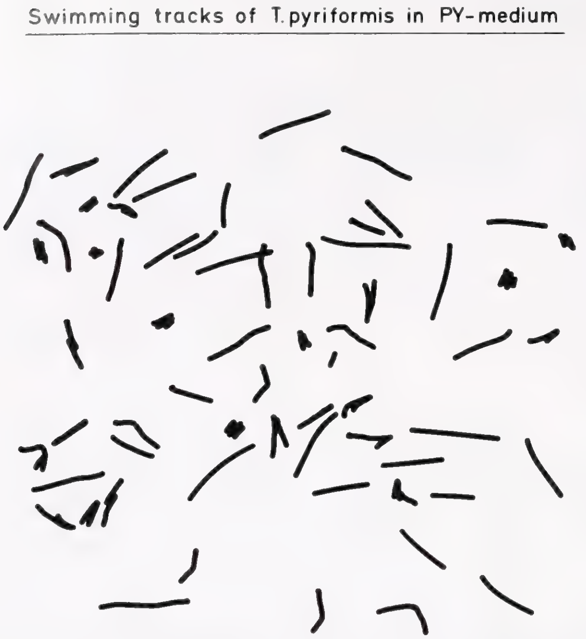

FIGURE 2. Swimming tracks. *Tetrahymena pyriformis* cells (4×10^4 cells/ml) logarithmically growing in PY-medium were placed under a coverslip supported by small coverslips to give a chamber with a depth of 300 μm. The cells (25°C) were examined with a Leitz Tass Image Analyzer at 7× magnification and swimming tracks were generated electronically (see Materials and Methods). The Figure shows a representative collection of tracks. For reasons of clarity only 63 tracks are shown here. Each track corresponds to the movement of a single cell within 1 s and serves as a basis for calculation of swimming velocities and for estimation of per cent cells showing 0, 1, 2 etc. turns within 1 s.

after addition of various compounds. Figure 2 shows a representative number of swimming tracks from cells growing logarithmically in PY-medium at 25°C. On the basis of the tracks in Figure 2 and analogous pictures for other conditions it was found that ~50% of the cells are "runners," *i.e.,* show no turns whereas ~25% show one turn within 1 s. The percentages for starved cells were 40% and 35%, respectively, in agreement with the results of Almagor *et al.* (1981). Additions to starved cells of amino acids or various proteins (*e.g.,* PDGF) were without effect on the percentage of runners.

To determine to what extent the chemosensory effect of PP is mediated by an increase in the swimming speed, this parameter was measured for starved cells (Tris control), for cells growing in PY-medium, and for various conditions where different compounds were added to starved cells (Fig. 3). The rationale for adding imidazole and IBMX was to decrease and increase, respectively, the intracellular concentration of cAMP (Wells *et al.,* 1975). The means of the distributions ± standard deviations (mm/s) were 0.40 ± 0.10 (+ imidazole), 0.42 ± 0.08 (control), 0.51 ± 0.11 (+PP), 0.52 ± 0.08 (PY-medium), 0.52 ± 0.11 (+dbcAMP), 0.63 ± 0.10 (+IBMX), and 0.63 ± 0.12 (+IBMX + dbcAMP). By applying *t*-tests on the random distributions we found significant differences ($P < 0.001$) for comparisons between the control value and all other values except that of imidazole ($.10 < P < .20$). Thus, the cells swim faster in PY than when starved and the addition of PP increases the swimming speed to the level of that of cells in PY. Interestingly, this speed value is also obtained by

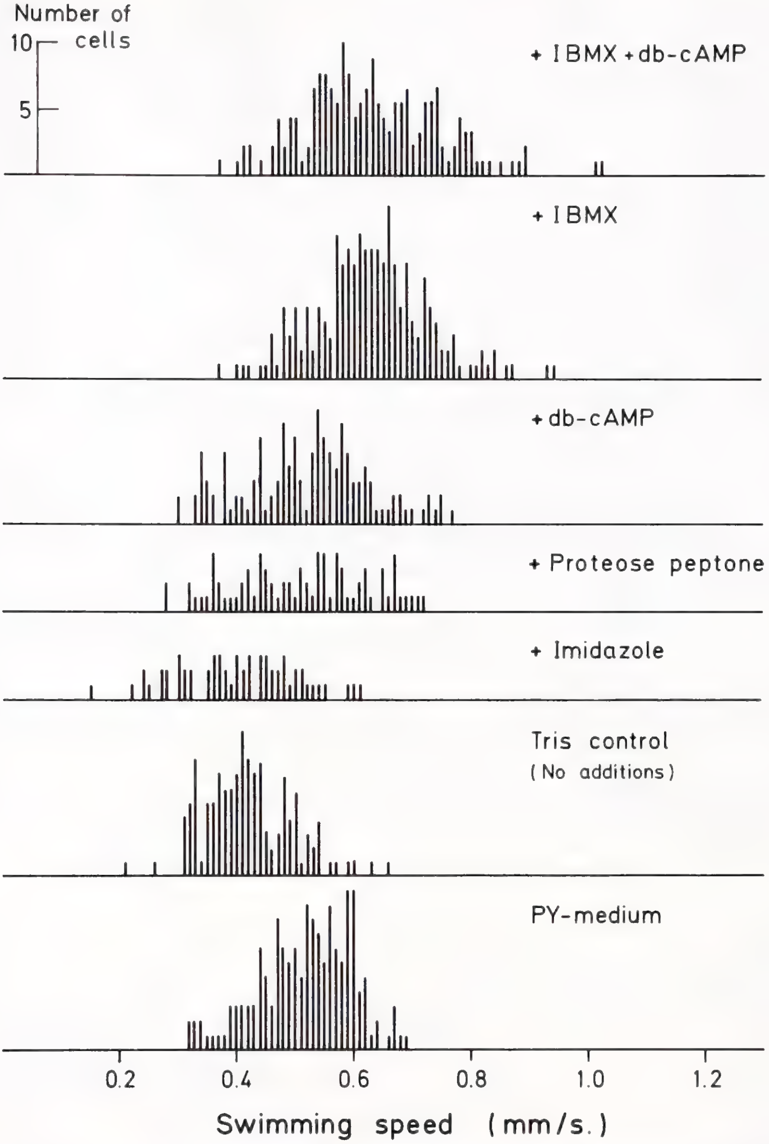

FIGURE 3. Distribution of swimming speed under different conditions. On the basis of the swimming tracks showing 0 turns (Fig. 2) the swimming speed was calculated for *T. pyriformis* cells grown in PY-medium (bottom profile) and for cells starved for 16 hours in 10 m*M* Tris-buffer, pH = 7.4 (Tris control). The other profiles show the effect (after 20 minutes) of addition of imidazole (10 m*M*), proteose peptone (0.1% ~ 300 μM), db-cAMP (1 μM), IBMX (1 m*M*), and IBMX + db-cAMP. The number of tracks analyzed was (from top to bottom) 170, 200, 130, 80, 60, 130, and 170.

addition of db-cAMP. IBMX gives a further increase in swimming speed, but this value cannot be further increased by addition of db-cAMP. Simultaneous addition of imidazole and IBMX (with 10-fold molar excess of imidazole) gives no effect. When IBMX is added to cells in PY or to starved cells with PP added, the swimming speed is increased to about 0.63 mm/s, which seems to be a maximal value for *Tetrahymena pyriformis* strain GL at 25°C.

As indicated in Table I the swimming speed was also measured after addition of PDGF (9 n*M*) (a concentration giving a significant chemosensory response), of EGF (20 μ*M*) (moderate chemosensory response), and of glucose (37 n*M*). None of these additions affect the swimming speed.

Table I also shows the effect of various additions on cAMP levels. It is evident that there is no general correlation between the swimming speed, the cAMP level, and the chemosensory response. Actually, most if not all of the situations seem to have their specific characteristics. Imidazole by itself is neither an attractant nor a repellent. Nandini-Kishore and Thompson (1979) found an 8-fold increase in cAMP level of *T. pyriformis* (stationary phase) by addition of glucose (37 m*M*). Apparently, compounds causing an increase in cAMP (db-cAMP, IBMX, and glucose) are not necessarily chemosensory stimuli and a positive chemoattraction is not necessarily accompanied by an increase in the swimming speed (PDGF and EGF).

The proteose peptone concentration 300 μ*M* (Table I) corresponds to 0.1% (assuming MW = 3.000). This concentration is supraoptimal for chemosensory response measured by the two-phase assay (Table II). We chose to use this assay because of its convenience and sensitivity to estimate the concentrations giving a 10%, a 50%, and a 100% response for various components—proteins and peptides (Table II) and amino acids and other components (Table II). One hundred percent (100%) response means that all cells have moved from upper to bottom phase within 20 minutes after set-up.

Table II shows that 9 μ*M* proteose peptone is sufficient for maximal response with the two-phase assay. Other peptides like EGF and EGF 3–14 are less active, whereas leukocyte chemotactic peptide is slightly more active. The peptides in YE give comparable results. Fractionation of the peptides in the heterogeneous mixtures of PP and YE by chromatography on Sephadex and HPLC showed that the peptides of medium length are more active than smaller or longer peptides (results not shown). In the case of PP the cells respond positively (and maximally) to concentrations between 9 μ*M*

TABLE I

Relation between the intracellular level of cAMP, the swimming velocity, and the chemoattraction of Tetrahymena pyriformis

	cAMP (pmoles/10^6 cells)	Swimming speed (mm/s)	Chemoattraction
Control	1.2 ± 0.2 (100)	0.42 ± 0.08 (100)	
Proteose peptone (300 μ*M*)	1.7 ± 0.3 (142)	0.51 ± 0.08 (121)	+
db-cAMP (1 μ*M*)	4.1 ± 0.3 (342)	0.52 ± 0.11 (124)	0
IBMX (1 m*M*)	2.8 ± 0.4 (233)	0.63 ± 0.10 (150)	−
Imidazole (10 m*M*)	1.6 ± 0.3 (133)	0.40 ± 0.10 (95)	0*
PDGF (9 nM)	n.d.	0.41 ± 0.09 (98)	+
EGF (20 μ*M*)	n.d.	0.41 ± 0.09 (98)	(+)
Glucose (37 m*M*)	n.d.	0.43 ± 0.08 (102)	0

Various compounds were added to cells starved in Tris-buffer for 16 h (control) (25°C). After 30 minutes aliquots were analyzed for level of cAMP (average of 4 separate experiments), swimming speed was measured at the same time (average of at least three separate experiments yielding 60–200 tracks). The results are given as mean (±S.D.). Chemoattraction was measured with the capillary assay, the gel assay, and the two-phase assay, which in all cases gave the same results. Numbers in parenthesis show percent compared to control. +: Attractant, −: Repellent.

* Imidazole abolishes the effect of IBMX when present in 10-fold molar excess.

TABLE II

Chemoattraction of starved Tetrahymena pyriformis *towards proteins and peptides. The two-phase assay was used*

		Concentration giving a certain response			
Compound	MW	10%	50%	100%	Comments
PDGF	30.000	1 n*M*	6 n*M*	30 n*M*	PDGF was kept as stock solution in 0.5 *M* HAc. An aliquot was neutralized with NaOH before use. The acetate by itself showed 10% attraction at 3 n*M* which is the conc. in PDGF at 30 n*M*.
Serum albumin fat-free (bovine)	68.000	4	n.d.	65	Excess albumin (265 n*M*) showed 90% response.
Histone 3 (calf thymus)	15.324	n.d.	65	110**	Excess histone 3 (600 n*M*) showed 0% response.
Histone 3 (calf thymus)	11.282	n.d.	45	90**	Excess histone 4 (800 n*M*) showed 0% response.
Serum albumin (human)	68.000	15	70	135	Excess albumin (300 n*M*) showed 100% response.
Lysozyme (egg white)	14.400	7	70	150	Excess lysozyme (1.2 *μM*) showed 0% response.
Hexokinase (yeast)	102.000	n.d.	50	200	
Cytochrome C (horse heart)	12.500	n.d.	160	400	Excess cytochrome C (1.6 *μM*) showed 80% response.
EGF (mouse)	6.045	3300	32000	n.d.	No effect of EGF at 330 n*M* and 33 n*M*.
EGF 3–14* (mouse)	1.310	4200	n.d.	n.d.	No effect at concentrations 420 n*M* and 42 n*M*.
Proteose peptone	3.000 average	1000	4500	9000	Excess PP showed 100% response at 1 m*M* and 0% response at 10 m*M*.
Leucocyte chemotactic peptide (f-met-leu-phe)	438	n.d.	2500	n.d.	Excess peptide (50 *μM*) showed 0% response.

n.d. = not determined.

* Part of EGF containing amino acids 3–14.

** Maximal response was consistently 90%.

and 1 m*M*, but they are not attracted by 10 m*M* (Table II). Such an optimal plateau of concentration is seen for the majority of attractants.

A number of proteins were tested and it is evident that all are considerably more active on a molar basis than the peptides EGF and PP (Table II). This is especially true for PDGF, but a number of other proteins are also active in the nanomolar range. We carefully checked the effect of salts and have found an attractant effect of acetate (10^{-4}–10^{-3} *M*). Therefore the effect of PDGF (stock in strong HAc) was corrected for the effect of acetate. A number of other anions (bicarbonate, sulphate, phosphate) showed no effect in concentrations 10^{-9}–10^{-3} *M*. No effect was observed with various lithium salts (10^{-5}–10^{-3} *M*) or with sodium vanadate (10^{-5}–10^{-3} *M*). It is noticeable that 4-fold excess of BSA and cytochrome C compared to their optimal concentration show [illegible] chemoattraction, whereas 5-fold and 8-fold excess of histone 3 and ly[illegible] respectively, show 0% response.

[illegible] of the amino acids (three of the nine tested) namely cysteine, methionine, and phenylalanine show a positive though not complete response at concentration of about 10^{-4} M (Table III). A mixture of 10 amino acids (10^{-4} M) consistently gave 100% response, but dilution to 10^{-5} M reduced the response to about 25%. A number of other low molecular weight substances were tested including carbohydrates and cyclic nucleotides. All of them showed no or only a slight response.

Measurements with the capillary assay have shown that proteose peptone (0.1%) attracts all of the cells (10^4/ml) in a 2 ml outer chamber (Leick and Hellung-Larsen, 1985). With the purpose of getting information about the mechanism behind this response four different experiments were performed (results not shown): (1) If the size of the outer chamber is increased to 15 ml, 40 ml, or 80 ml, and the cell concentration is kept constant at 10^4 cells/ml, the *absolute* amounts of cells trapped were 60, 70, and 80 × 10^3 cells, respectively. (2) If IBMX (1 mM) is added to the outer chamber the cells accumulate in the trap since IBMX is a repellent. (3) If IBMX (1 mM) is added to inner and the outer chamber with PP present in the inner chamber the time necessary for complete accumulation of cells is decreased. (4) Only 7% of color dye (bromophenol blue) diffuses out of the inner chamber during 24 hours (spectrophotometric measurement). These capillary apparatus results seem to indicate that (a) short-ranged concentration gradients are formed close to the outlets of the capillaries and (b) the accumulation of cells is strongly dependent on the swimming speed of the cells in the outer chamber, because the gradient is short compared to the dimensions of the outer chamber.

TABLE III

Chemoattraction by starved Tetrahymena pyriformis *towards amino acids and other low molecular weight substances. The two-phase assay was used*

Compound	Optimal concentration range (M)	Response
Asp	10^{-4}–5×10^{-4}	Weak*
Citrullin	2×10^{-4}–10^{-3}	Negative
Cys	2×10^{-4}–2×10^{-3}	Positive
His	2×10^{-4}–2×10^{-3}	Weak
Ile	2×10^{-4}–10^{-3}	Negative
Leu	2×10^{-4}–10^{-3}	Negative
Met	2×10^{-4}–10^{-3}	Positive
Phe	2×10^{-4}–2×10^{-3}	Positive
Thr	2×10^{-4}–5×10^{-3}	Weak
Trp	2×10^{-4}–10^{-3}	Negative
Mixture of amino acids**	10^{-4} of each	Positive
Betaine	2×10^{-4}–10^{-3}	Weak
Cadaverin	2×10^{-4}–10^{-2}	Weak
Dibuturyl-cAMP	10^{-4}–10^{-3}	Negative
Dibuturyl-cGMP	10^{-4}–10^{-3}	Negative
Glucose	10^{-4}–10^{-2}	Negative
Glutathione	2×10^{-4}–2×10^{-3}	Weak
Glycerol	10^{-3}–10^{-2}	Negative
Glycerophosphate sodium salt	10^{-3}–10^{-2}	Negative
Sucrose	10^{-3}–10^{-2}	Negative
Thymine	10^{-3}–10^{-2}	Negative

* Weak: accumulation after 10–20 min of 5–20% of the cells initially present in the upper phase.

** Contains ala, arg, asn, glu, gln, gly, his, ile, leu, lys, met, phe, pro, ser, thr, trp, and val.

With the field assay (Table IV) it was possible to answer some important questions: how rapidly do the cells respond to various chemical stimuli? Do they accelerate on their way towards the attractant? Is the swimming speed greater towards than away from the attractant? The results are shown in Table IV. At time 0 there is the same number of cells in all fields but after only 1 min the cells accumulate in field 1. Apparently some cells are drained from fields 2 and 3, others enter from areas outside the scanned area, which had a width of 2.1 mm.

As indicated in the lower part of Table IV the cells in field 10 swim 0.38 mm/s [comparable to the control value for starved cells (0.42) in Figure 2]. In field 6 where accumulation of cells is about to increase (720 after 16 min) the swimming speed is 0.44 mm/s. Thus, the speed increases with decreasing distance to the attractant. After 12 minutes the swimming speed was measured in field 2. The cells moving towards the attractant have a higher speed than those swimming away from it. The values differed significantly ($P < 0.001$). In some experiments we used Ultroser G (a mixture of proteins such as adhesion and growth factors) as an attractant instead of PP. The results became more reproducible with Ultroser G probably due to the fact that the proteins in Ultroser G diffuse more slowly out of the gel than the small peptides in PP.

DISCUSSION

The main purpose of the present paper was to elucidate the relative role of chemokinesis in chemoattraction of *Tetrahymena pyriformis,* Strain GL. We have confirmed that the turning/tumbling frequency may be modulated by the presence of attractants and repellents (Almagor *et al.,* 1981) and that the swimming speed may be increased when the cells approach the attractant (Levandowsky *et al.,* 1984). How-

TABLE IV

Analyses of cell accumulation and swimming speed of Tetrahymena pyriformis *as a function of distance from attractant*

Field number		1	2	3	6	10
Mean distance from attractant (mm)		1.2	3.6	6.0	13.2	22.8
Cell number per field at various times after addition of cells	0 min	600	600	510	600	600
	1 min	1350	580	510	n.d.	n.d.
	2 min	1920	550	380	n.d.	n.d.
	12 min	$\gg$1920	1730	450	n.d.	n.d.
	16 min	$\gg$1920	$\gg$1920	1720	720	610
Swimming speed (mm/s.) 12 min after addition of cells		0.55	0.56 ± 0.07 (towards attractant) 0.49 ± 0.06 (away from attractant)		0.44	0.38

A chamber was made on top of a glass slide by means of piece of agarose gel (1% HSA agarose in 10 m*M* Tris, pH = 7.4) and a coverslip to give a depth of 600 μm. One piece of gel containing attractant (0.5% PP in Tris-buffer) was placed close to the edge of the coverslip thereby allowing sufficient access to oxygen. Cells starved in Tris buffer overnight (2×10^5 cells/ml) were added under the coverslip (25°C). The cell number and the swimming speed were registered in fields (2.4 mm × 2.1 mm) with a certain distance from the gel with attractant. The swimming speed was based on the length of tracks during a period of 1 s. A number of 50 tracks was analyzed per calculation. The results are given as mean (±S.D.).

ev[illegible] observations have been extended in the following way: PP influences the [illegible] speed—probably due to cAMP concentration—whereas other attractants [illegible] (Table I). These data are in reasonable agreement with those of Voichick *et al.* [illegible] Nandini-Kishore and Thompson (1979). We have been aware of the fact [illegible] swimming speed is strongly dependent on the culture temperature (Hill, 1983). The chemosensory attraction of *T. pyriformis* is not only seen with starved cells. Actually cells in DM respond towards PP, YE, and PDGF, and cells in PY-medium respond towards PDGF.

Interestingly, the optimal concentration of PP for growth is not the same as that for chemoattraction. The same holds true for the amino acids of DM. In both cases the optimal chemoattraction is seen at a 10–50 fold lower concentration than that in the growth media.

The role of chemokinesis is obvious from the measurements of swimming speed after various additions (Table I) and in different distances from an attractant (Table IV). Values for swimming speed at 25°C (0.40–0.63 mm/s.) are comparable to those reported by Hill (1983) and Levandowsky *et al.* (1984) for *T. thermophila.* The correlation of increase in swimming speed to increase in cAMP level seems to be positive (Table I).

By use of the capillary assay, we found earlier with *T. thermophila,* B7, that a mixture of amino acids show chemoattraction (Leick and Hellung-Larsen, 1985). The two-phase assay used in the present study allows us to conclude that for *T. pyriformis* cysteine, methionine, and phenylalanine are attractants, whereas histidine, isoleucine, leucine, and threonine, and tryptophan are not. Levandowsky *et al.* (1984), using *T. thermophila,* CU strain 307, found cysteine, methionine, histidine, and leucine to be positive. Almagor *et al.* (1981), using *T. thermophila,* strain WH 52, scored leucine and methionine as positive. We have no explanation for the differences, but we have at least confirmed that an interesting specificity exists for some of the amino acids. Furthermore, the present data indicate that the amino acids (on a molar basis) are less active than peptides and proteins. The peptides in PP and YE are good attractants in micromolar concentration. Apparently those of middle-length are most active, but all peptides tested so far show some chemoattraction. Proteins are on a molar basis the most efficient chemoattractants and considerable specificity exists (Table II). Thus, 6 n*M* PDGF gives 50% response whereas about a 10-fold higher concentration is needed for histones 3 and 4.

Attempts to demonstrate receptors in *Tetrahymena* for PDGF by use of ^{125}I-PDGF (Heldin *et al.,* 1981), to induce tyrosine-specific phosphorylation by PDGF (Ek and Heldin, 1982), and to measure PDGF and EGF in extracts or exudates of cells by a radioreceptor assay thus far have been unsuccessful (Heldin and Hellung-Larsen, unpub.).

ACKNOWLEDGMENTS

The skilled technical assistance of Inger Lyhne is gratefully acknowledged. Dr. Helge Andersen made valuable suggestions concerning the manuscript, which was typed by Ellen Høyer. EGF was kindly supplied by Dr. Jack Johansen, Carlbiotech, Copenhagen, and PDGF was a generous gift from Dr. C.-J. Heldin, Uppsala, Sweden.

The work was supported by grants from Lykfeldt's Legat and the Danish Medical Research Council.

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MIGRATION IN THE GRASSHOPPER *MELANOPLUS SANGUINIPES* (FAB.). I. THE CAPACITY FOR FLIGHT IN NON-SWARMING POPULATIONS

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ABSTRACT

A tethered flight assay which identifies migratory behavior in the grasshopper *Melanoplus sanguinipes* (Fab.) was developed. The validity of the assay was examined by flight testing individuals collected at random from migratory and from virtually non-migratory field populations. Behavior in the tethered assay was found to accurately reflect the relative amount of migratory behavior in the field populations. Migratory flight appears to occur in this species even in the absence of obvious swarming behavior. Interpopulation differences in flight behavior observed in the field insects were maintained in first generation laboratory-reared progeny. Results of this study imply that migration is likely to play a significant role in the ecology of *M. sanguinipes,* even in nonoutbreak populations, and is not likely to be a simple facultative response to unusual environmental conditions.

INTRODUCTION

Migration has an integral role in the ecology of many species (Baker, 1978; Dingle, 1980; Lidicker and Caldwell, 1982). It may be an important factor limiting species range and distribution (Andrewartha and Birch, 1954) and is necessary if species are to invade and colonize new habitats. The frequency of migration is a major determinant of the rate of gene flow between two populations, and dispersal can stabilize populations in heterogeneous environments (Roff, 1974).

Understanding the significance of migration in a species' ecology requires a means of assessing its frequency of occurrence. However, with notable exceptions such as flights of the Monarch butterfly and swarms of the Middle Eastern locusts, the migrations of insects in the field are usually not apparent, and detection of long distance movement may require extraordinary effort. Even when insect movements are obvious or when attempts are made to assess the number of migrant individuals in a population, impressions of the extent of migration may be erroneous. For instance, reliance solely on observation of mass swarms alone can underestimate the frequency of migration in a species; individuals in nonswarming populations of the locusts *Schistocerca gregaria* (Forskal) and *Locusta migratoria* (R. et F.) also migrate (Davey, 1955, 1959; Roffey, 1963). Mark-recapture studies can provide information about the orientation and distances of movement, but may seriously underestimate the extent of migration if loss of individuals from the study population by death cannot be distinguished from loss due to emigration or if the appropriate life stage for migration is not chosen for study (Mallet, 1984). The difficulties of recapturing migrants that have moved significant distances from the point of release are compounded by a "dilution effect" as the area into which the organism has emigrated increases with the square of the distance

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Received [illegible] August 1984; accepted 25 March 1986.

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moved (Southwood, 1966). Mark-recapture and other methods of identifying migrant insects that have been used in the field, such as radar detection or various means of trapping, are usually not practical for laboratory studies of such behavior.

The North American grasshopper, *Melanoplus sanguinipes* (Fab.), periodically engages in spectacular long distance mass migrations that can have serious economic impact on crops and rangeland. This swarming behavior, however, has been observed only rarely since the early 1900's and appears to be associated with unusual environmental conditions (Shotwell, 1930; Parker *et al.*, 1955; Scharff, 1961; Pfadt, 1982). Mark-recapture studies have suggested that nonswarming populations of *M. sanguinipes* are relatively sedentary (Riegert *et al.*, 1954; Baldwin *et al.*, 1958; Edwards, 1961). However, in these studies, recapture rates were quite low, and the authors could not distinguish death (with rapid removal of remains by scavengers) from emigration beyond the recapture search range. These studies implied that migration is extremely infrequent in this species, occurring only under extreme conditions. Based on such findings, it would seem that in the ecology of *M. sanguinipes* migration serves primarily as a means of escape from unfavorable conditions. We suggest, however, that earlier studies have greatly underestimated the frequency of movement in this species because of limitations of the field methods used to obtain estimates of migration. It is therefore desirable to attempt to examine the degree of migration in this species using a combination of field and laboratory methods. To that end we have employed a tethered flight assay in combination with field observation and have found that individuals migrate much more frequently than previous reports indicated. Indeed, although the proportion of migrants can vary greatly between populations, our results show that some individuals in all non-swarming populations may be migratory.

MATERIALS AND METHODS

Flight assay

The tethered flight assay of Dingle (1965) was modified for use with *M. sanguinipes.* Grasshoppers were attached at the dorsal pronotum to a small lightweight stick using warmed Styx brand wax. Insects were suspended from a horizontal bar facing a fan generating a breeze of 11–13 km/h. Floodlights were used to increase the illumination and to raise the air temperature at the flight test station to 32–34°C. Flight tests were conducted between 3 and 12 hours after sunrise or lights-on. These conditions simulate those observed to be necessary for occurrence of migratory flights in the field (Parker *et al.*, 1955; Bird *et al.*, 1966) and flight in a wind tunnel (Riegert, 1962). Take-off was initiated by breaking tarsal contact or by moving the tethered animal rapidly through the air in a horizontal figure-8 pattern for several seconds. Test insects were allowed a maximum of five such take-off simulations or restarts during the flight test period (see Results). Total cumulative flight time was recorded. Occasionally, grasshoppers would hold wings outstretched without flapping. Only movement of both fore and hindwings was scored as flight.

Insects were flight tested within 24 hours of capture. Colorado field flight tests were conducted in a small mobile field lab. Arizona field flight tests were conducted either in a USDA facility in Phoenix or at the Bureau of Indian Affairs–Branch of Forestry in San Carlos.

Field collection and laboratory maintenance of grasshoppers

Insects used in field flight tests were collected from two areas in late July and early August. The first collecting area was a site 8.8 km north of Ted's Place (approximately

40[illegible] [illegible]5°10′W), Colorado (the CO population) along US Highway 287. The [illegible] an area approximately 1.6 km northeast of Cassadora Springs (approxi[illegible]4′N and 110°20′W) on the San Carlos Apache Indian Reservation, Arizona [illegible] population). Determination of the degree of migration at each site was based on published reports, observation of sustained flight, and behavior after release from confinement (described below). Grasshoppers from each field population were shipped to the University of Texas at Austin and used to establish separate colonies. Insects were reared in 30 × 30 × 30 cm screen and Plexiglas cages placed in Hotpack walk-in environmental chambers at 16L:8D photoperiod, 30–50% relative humidity, and 30°C. Fresh, washed lettuce and a mixture of 50:50 bran and wheat germ were available continuously. Growth chambers and cages were rinsed after each experiment in a 5.5% bleach solution followed by thorough hot water rinse. Cage design allowed feces and debris to drop through a screen floor onto a mat which was changed daily.

Containers of damp, sterilized sand were provided for oviposition. Egg pods were sifted from the sand and placed in moist vermiculite for at least 28 days at 30–32°C. Any eggs that had not hatched at the end of this incubation period and were not obviously diseased were assumed to be in diapause (Salt, 1949; Riegert, 1961) and placed in a cold room at 4–8°C for at least 3 months.

RESULTS

Criteria for migratory flight in the laboratory tethered assay

To establish a practical laboratory assay for migratory behavior in *M. sanguinipes,* the tendency of AZ laboratory-reared individuals to initiate and maintain tethered flight was assessed. Each insect was allowed to fly until stopping itself, up to a maximum flight duration of 4 hours. Flight was monitored at 5-minute intervals (*i.e.,* 60 minutes represents animals that flew 56–60 min). Cuticle hardening was sufficient to allow flight by day 2 after adult emergence.

Insects ranged in age between 2–15 days post-eclosion and were tested on 2–3 separate occasions at least 3 days apart. In this experiment, performance in one flight test had little predictive value in a subsequent trial ($r^2 = 0.077$). Therefore the trials were assumed to be independent. The flight duration curve for the population was markedly skewed and is approximated by two distinct lines which intersect at the 60-minute interval (Fig. 1). Once the insect had flown 60 minutes it was much more likely to continue; 55% of the flights ended before the first hour, while 78% of the flights in progress at 60 min continued through the next hour. Although we have observed sustained flights of longer than 10 hours, we have not established an upper limit for flight duration in this species. As previously mentioned, grasshoppers were allowed a maximum of 5 take-off simulations during flight testing. This was necessary in part to compensate for disturbance of flight produced by the investigator when new grasshoppers were placed on the flight testing apparatus, although grasshoppers would also occasionally stop spontaneously. Short (less than 5 min) and intermediate (6–55 min) flyers stopped and were restarted more frequently ($x = 2.65$ times) before refusing to resume flight. Long flyers (flight longer than 60 min) stopped only an average of 1.25 times before cessation of flight (differences significant at $P < 0.001$, one-tailed t test). Thus, grasshoppers making flights in excess of 60 minutes were not only more persistent, but also were less easily interrupted than those making shorter flights. A flight time of 60 min or more is long enough for movement over a considerable distance or to reach regions of the atmosphere where transport by wind currents can occur (see Johnson, 1969). Sixty-minute flights are also quite distinct from the animal's

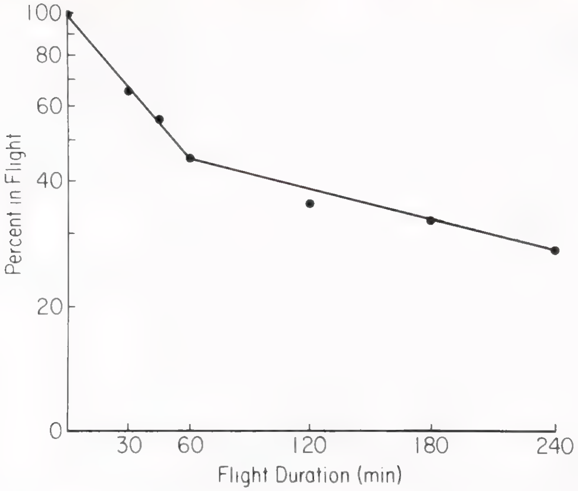

FIGURE 1. Semi-log plot of the percent of grasshoppers still in flight at given duration. Lines were drawn by sight. Data were recorded in 5-min intervals, *i.e.*, the 60-min point represents flights of 56 to 60 minutes. Flights still in progress at 4 hours were terminated. n = 16 males and 16 females with a total of 66 flights.

normal locomotor responses to vegetative or escape stimuli that usually evoke walking, hopping, or very brief flights of less than 2–3 seconds duration. Therefore, a flight duration of 60 minutes was chosen as the criterion for migratory behavior in *M. sanguinipes*, and in subsequent tests insects were stopped after they had flown one hour. The criterion was challenged by flight testing animals from field populations that exhibited different degrees of migratory behavior (see below).

Classification of field populations

Assessment of the migratory tendencies of field populations was based on several lines of evidence.

Published reports. Mass migrations had been observed at the AZ collecting area in early summer in both 1980 (Pfadt, 1982) and 1981 (Foster, pers. comm.), and marching hopper bands, characteristic of migratory locusts (Uvarov, 1966), were observed in 1980 and 1981 (Foster, pers. comm.).

There were no published reports of such activity at the CO site, although grasshopper populations had been monitored in 1980 and 1981 in conjunction with rangeland treatment programs.

Field observations of spontaneous flight activity. In the first week of August 1981 and in the last two weeks of July 1982, much spontaneous flight activity was observed in the AZ population during mid-day (10:00–16:00). The grasshoppers moved too rapidly to follow on foot. However, by standing motionless in an infested area (considered by agricultural entomologists to be a region with grasshopper densities = $8/\text{yd}^2$ or $8.81/\text{m}^2$) and scanning the horizon with 15× binoculars, we were able to detect sustained flights of at least 6 m above the ground which lasted more than 60 s and which carried the insect at least 50 m (beyond binocular range). These represent minimum possible estimates of the duration and distances covered by individuals; actual time in flight was certainly much greater. In contrast, at the CO site during two weeks each in late July 1980 and 1981 and two weeks at the end of July and beginning of [illegible] 1982, sustained flights were not observed either while surveying the population with binoculars or during sweep netting. The latter activity stimulated some movement of grasshoppers including escape flights but these lasted no more than 2–[illegible] carried the insect at most 3 m. Attempts were made to quantify movement more precisely in each population with malaise net trapping and mark-recapture studies. However, the malaise trap proved to be an ineffective method for collecting flying grasshoppers and recapture rates were far too low to provide any estimate of death or emigration at either site.

Locusts, especially in the solitarious state, often migrate after sunset (Davey, 1955, 1959; Roffey, 1963), and, *a priori,* the high daytime temperatures and possible water loss problems at the AZ site would seem to favor nocturnal flight. Tethered flight tests made without lights during the animals' scotophase indicated that *M. sanguinipes* is capable of flight in the dark (McAnelly, 1984). However, in the course of nighttime observations either aided by flashlight or made when light levels (illumination from moonlight) were sufficient to detect small flying insects and to identify stationary and crawling insects, no movement of *M. sanguinipes* was detected in either field population. Although collections and scanning of fields with binoculars were done immediately after sunset and throughout the night, grasshoppers were quite immobile, usually roosting on vegetation. It may be that the temperature threshold for flight in this species is too high for nocturnal flight to occur frequently. Average daily minimum temperatures for July and August were less than 21.5°C in AZ and less than 15°C in CO (BIA-Forestry, San Carlos and Colorado Climate Center data). However, our observation period was quite limited (a total of 3 nights in AZ and 2 nights in CO), and we do not rule out the possibility of nocturnal flight in *M. sanguinipes* under appropriate conditions.

Flight of released animals. At each field site, resident grasshoppers were collected ($n =$ approximately 40 at both sites) and confined in screen cages (with food available) for 3 days and then released on warm sunny days with light breeze. When AZ insects were released, all actively took flight and approximately 10% continued the flight beyond the range of binocular sight (50 m). No CO insects flew more than 1.0 m upon release while most slowly crawled out of the cage. Ambient temperature was well above flight threshold (29.4°C–Riegert, 1962) in both cases, though somewhat higher (41°C) in AZ than in CO (37°C).

Census data. Census data also revealed evidence of large scale population movements in AZ. At a site 1.6 km from Cassadora Springs in which we observed considerable spontaneous take-off activity, density was reduced from approximately 20 *M. sanguinipes*/m^2 (estimated by counting the number of grasshoppers flushed/m^2) to less than 1 grasshopper/m^2 within a 2-day interval. We observed no evidence of increased mortality or predation during this interval, suggesting almost total emigration from this site. Similar changes in local population density were not observed at the CO site (densities over a three-day period in one area averaged $13.3/m^2 \pm 3.3/m^2$).

Results of field flight tests

The validity of the stationary tethered flight assay for *M. sanguinipes* was tested by comparing flight performance of grasshoppers from the two field populations. Tethered flight tests conducted on field-collected animals revealed a highly significant difference in flight behavior between the AZ and the CO animals with AZ insects showing a much greater tendency to make long flights than CO animals (Table I). These results were consistent over a two-year period. Subdividing the 2×3 Chi square analysis for 1982 data into a 2×2 Chi square of short and intermediate categories (Snedecor and

TABLE I

Comparison of the flight performance of CO and AZ field populations in the stationary tethered flight assay

	1981		1982	
Flight duration (minutes)	AZ	CO	AZ	CO
0–5	17 (57%)	130 (86%)	43 (28%)	72 (49%)
6–55	7 (23%)	17 (11%)	66 (43%)	69 (47%)
56–60	6 (20%)	4 (3%)	43 (28%)	7 (5%)
Total	30	151	152	148
Percent migrants	20	3	28	5

Figures in parenthesis indicate the percent of total in each category (rounded to nearest whole percent).
1981: $2 \times 3\chi^2$ for 0–5 *versus* 6–55 *versus* 56–60 minute flights = 18.55, $P < 0.001$.
1982: $2 \times 3\chi^2$ for 0–5 *versus* 6–55 *versus* 56–60 minute flights = 33.27, $P \ll 0.001$. $2 \times 2\chi^2$ for 0–5 *versus* 6–55 minute flights = 3.35, $P > 0.05$.

Cochran, 1967) highlights the proportionately smaller contribution of the differences between short and intermediate flights (6–55 min) to the overall differences between populations. Therefore the main component of the difference in flight behavior between populations was, in fact, that due to flights longer than 60 min.

Flight behavior of laboratory-reared progeny

Difference in the flight behavior between the AZ and CO field populations has potentially important implications for the ability of this species to adapt to local conditions. However, it is first necessary to ascertain that the difference is actually due to interpopulation variability and not simply to differences in sampling. For instance, the decreased flight behavior observed in the CO population relative to the AZ population might be explained by differences in age or reproductive status of individuals at the two sites. Although exact age of the field animals could not be determined, dissection of field tested grasshoppers, indicated that reproductive differences did not account for behavioral differences (McAnelly and Rankin, 1986). Furthermore, the interpopulation differences observed in the field were retained in progeny reared under identical conditions in the laboratory (Table II). In this experiment, each individual was flight tested every day from day 2 post-eclosion until day 28. Both the number of "migrants" (defined as individuals which made at least one 60-minute flight) and the percentage of long flights made by "migrants" were determined. Significantly more AZ than CO grasshoppers were classified as "migrants" (Table IIA), and the AZ "migrants" made long flights significantly more frequently than those from the CO line (Table IIB). These results indicated that the interpopulation differences in migratory behavior are persistent and that short-term differences in environmental conditions between the AZ and CO habitats were not sufficient to account for the observed differences in migration.

Repeated laboratory flight testing identified a greater number of migrants than did single flight tests in the field for both populations (Table IIA). This would be expected when flight behavior of individuals is recurrent and variable over time (Davis, 1980). In this experiment, the percent of AZ or CO individuals making long

TABLE II

ance of first generation lab-reared progeny from AZ and CO populations.
s were tested every other day from day 2 to day 28

Number of long flights	AZ	CO
0	16	27
1+	24	13
Percent migrants	60.0	32.5

$\chi^2 = 6.08, P < 0.02$

A. Number of individuals making a long flight at least once. No difference was observed between the proportion of males and females that made at least one 60-minute flight. Therefore data from both sexes were combined. Nineteen female and 21 male AZ progeny and 18 female and 22 male CO progeny were tested.

	AZ	CO
Median score (%) of long flights in all trials made by "migrant" individuals	35.4	14.3
Range of scores	7.1–100%	7.7–66.7%
n	24	13

$z = 2.56, P = 0.0052$

B. Comparison of the repeatability of long flight behavior of "migrants" from AZ and CO laboratory-reared progeny. The percent of long flights in all flight trials made by each individual is treated as a flight score. The scores for AZ and CO "migrants" were ranked and compared using the Mann-Whitney U for large samples, corrected for ties (Siegel, 1956). Within a population, there was no difference between males and females with regard to the percentage of long flights made by "migrant" individuals. Therefore data from both sexes were combined.

flights on any given day was similar to the percent recorded in single flight tests of the respective field populations. As a practical consideration in designing future experiments, we found that approximately 90% of the total number of migrants identified in 14 laboratory flight trials would have been identified in the first 5 trials.

DISCUSSION

Tethered flight assays have provided some of the best corroborating evidence of field observations. Long duration tethered flight has been correlated with evidence of migration in the field for several insect species (Dingle, 1965; Rose, 1972; Rankin and Rankin, 1980). Further, *Tetraopes* beetles caught in flight between two habitats performed significantly better in tethered flight tests than insects collected on the ground (Davis, 1981). However, to avoid circularity it is necessary that independent criteria of field migration are used in devising and verifying a laboratory assay for migratory behavior.

The most direct field test of a laboratory flight assay would be the flight testing of insects actually caught in the process of migrating as Davis (1981) has done. We were not able to collect grasshoppers in migratory flight in the current study, although this may be possible in the future if field conditions are favorable. However, several different lines of evidence were brought to bear on the problem of determining the relative degree of migration in two populations. Because *M. sanguinipes* is a serious crop pest, populations are monitored regularly in both Colorado and Arizona. Thus we had independent assessments of the migratory tendency of both of our field populations for 1980–1982 in addition to our own observations. All of these criteria indicated a major difference in the amount of long-distance movement occurring in the two populations. Thus, these populations provided a real contrast in flight propensity with which to verify our laboratory assay.

Although the tethered assay clearly reflected the relative differences in flight behavior between AZ and CO insects, the exact correspondence between frequency of long-duration flight in the field and in tethered flight tests is difficult to assess. Some aspects of laboratory testing tend to overestimate while others underestimate actual flight in a field situation. For example, the proximate cues of warm temperature, light, wind speed (see Materials and Methods) known to be necessary for migration in both populations (Parker *et al.,* 1955; Riegert, 1962; McAnelly unpub. obs.) were provided during all flight tests, whereas field conditions are likely to be much more variable. On the other hand, in the field animals have the opportunity to fly any time conditions are appropriate, rather than only once every 2 or 3 days as in these experiments. Due to daily variability in individual flight performance, the likelihood that an insect will migrate increases with opportunity for flight. Thus given suitable conditions for flight every day, field animals might make migratory flights more frequently than indicated by the laboratory assay. Conversely, the flight test includes the artificial impetus of a simulated take-off which could inflate the laboratory estimate by obviating any take-off threshold that might limit numbers of migrants in the field. Analysis of the tethered flight behavior of grasshoppers actually caught in migratory flight may eventually allow more precise quantitative estimates of the numbers of field migrants.

Both field observations and flight assay results indicated that there were substantial numbers of migrant individuals in the AZ population even in the absence of mass swarming. The fact that flight test results from the CO population identified 3–5% of the individuals as migrants underscores the difficulty, discussed previously, of relying solely on field observations to estimate the numbers of migrants and suggests that even in a relatively sedentary population of *M. sanguinipes* there are some migratory individuals. Observations of *M. sanguinipes* in Colorado at altitudes above its normal range or trapped in snowbanks have been made annually and support the conclusion that a fraction of the CO population is migratory even in non-outbreak years (Alexander, 1964).

The finding that there are striking differences in migratory behavior between populations has important implications for any study of the biology of *M. sanguinipes.* The interpopulation variation in migratory behavior between the AZ and CO populations is associated with marked differences in habitat quality between the two sites. The high degree of migratory activity at the AZ site is likely to be a response to semi-arid conditions and patchy distribution of resources at this site (Southwood, 1962; McAnelly, 1984, 1985). The CO field and lab insects were clearly less migratory than the respective AZ grasshoppers, presumably reflecting the relatively more lush and uniform habitat at the CO site (McAnelly, 1984). Alexander (1964) has suggested that the limited movements of individuals to high-altitude Colorado habitats that are only occasionally suitable for completion of the life cycle serve to allow immediate expansion of range during favorable years.

The causal basis for these differences has important consequences for the means by which mass swarms develop and for the ability of this species to invade and exploit new habitats. Retention of differences in flight propensity in the first generation laboratory-reared progeny suggests that immediate environmental conditions experienced by the individual are insufficient to explain the interpopulation variation in migration.

The experiments described in this study do not allow us to distinguish between the relative influence of environmental factors experienced by the parents (*i.e.,* maternal effects) and the role of genotype in determining whether an individual becomes a migrant. However, subsequent work has indicated that migratory behavior is under strong genetic control in this species (McAnelly, 1984, 1985; Rankin *et al.,* 1986; McAnelly and Rankin, in prep.), suggesting that the geographic variation in flight behavior and temporal changes in the proportions of migrants within a population may be at least in part the result of differential selection. Thus, even a very low proportion of migrants could play a significant role in the biology of the CO population by providing the necessary variation upon which selection could act to shift the balance toward a greater degree of migratory behavior as environmental conditions change over time (McAnelly and Rankin, in prep.; Parker *et al.,* 1955; see also Danks, 1983).

ACKNOWLEDGMENTS

Our sincere thanks to M. E. Smyers and Dr. G. J. Steck who provided valuable field assistance. We thank the residents of the San Carlos Apache Indian Reservation, AZ, for permission to collect insects and Dr. N. Foster of the USDA-APHIS in Phoenix, AZ, and Mr. D. Reinhardt of the BIA–Branch of Forestry for kindly allowing us the use of facilities for flight testing. We are indebted to Dr. R. Pfadt of the University of Wyoming for information which was most useful in helping to locate suitable field sites. A very special thanks to Mr. and Mrs. Tom Russell who provided us with camping space and friendship during our work in Colorado and to Edward L. Richmond for the use of his truck and trailer as field vehicles. Drs. M. C. Singer, Darrell Moore, and Harold Zakon provided helpful criticisms of an earlier version of this manuscript. This work was supported by NSF grant–PCM 8110568 to Dr. Rankin and by University of Texas graduate student research scholarships to M. L. McAnelly.

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TION IN THE GRASSHOPPER *MELANOPLUS SANGUINIPES* II. INTERACTIONS BETWEEN FLIGHT AND REPRODUCTION

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ABSTRACT

Prolonged flight behavior in females of the grasshopper, *Melanoplus sanguinipes* (Fab.), decreases as ovarian tract weight increases relative to body weight. However, reproductive maturation does not always permanently terminate the migratory phase of the grasshopper's life cycle; long flights are also made after ovipositions. We examine the hypothesis that migration (measured as long-duration tethered flight behavior) decreases reproductive potential, either by delaying first oviposition or by decreasing production of egg pods. No negative effects of flight on oviposition were observed. Instead, age at first oviposition tended to decrease in females flown repeatedly compared to those flown only twice. Further, oviposition was greatly enhanced in females flown for several hours relative to unflown controls. We suggest that selection pressure for rapid and successful colonization has resulted in the evolution of a suite of characters including enhanced migratory behavior and accelerated reproduction in highly migratory populations.

INTRODUCTION

The relationship between migration and reproduction in insects can be analyzed from two perspectives: (1) the effect of migration on reproductive output, often seen as the reproductive cost of migration, and (2) the effect of reproductive development on flight behavior, which in some insects takes the form of an oogenesis-flight syndrome (Johnson, 1969) (suppression of long-duration flight behavior in females with mature ovaries). This paper examines these types of interactions between long-duration, presumed migratory flight behavior and reproduction in three populations of the migratory grasshopper *Melanoplus sanguinipes* (Fab.).

Migration is an important component of many insect life history strategies and can have profound impact on individual fitness (Dingle, 1972; Denno and Dingle, 1981). For example, it may allow escape from unfavorable environmental conditions, exploitation of two or more different kinds of habitats, or colonization of new areas (Southwood, 1962; see Lidicker and Caldwell, 1982; see Rankin and Singer, 1984). Interactions between migration and other life history characteristics, particularly those involving reproduction, also can have a major effect on fitness. Age at first oviposition, the timing of subsequent ovipositions, and total reproductive output, for instance, may be influenced by migratory activity. When apterae or nonmigrants are compared with their migratory conspecifics or members of closely related migratory species, negative effects of migration on reproduction are often evident. The sacrifice exacted by migration or possession of wings can be measured in slower nymphal development, increased time to first oviposition, decreased numbers of developed ovarioles, decreased numbers of eggs per clutch, or decreased overall fecundity (Dixon and Wratten, 1971;

Received 5 February 1986; accepted 25 March 1986.

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Dixon, 1972; Roff, 1977, 1984; Walters and Dixon, 1983; Zera, 1984, 1985). As a consequence, it is often assumed that migrants in general have decreased reproductive fitness relative to nonmigrants (Grinnell, 1922; Elton, 1936; Dingle, 1972; Roff, 1975, 1977).

Any reproductive costs to the individual resulting from migration must be taken into account in theoretical considerations of the evolution and maintenance of migratory behavior. However, the issue of reproductive cost is complex. It would be unrealistic to focus on decrements in reproductive output suffered by winged or migrant individuals while overlooking both the cost of not migrating and the long-term benefits to colonists. In considering the likelihood of the evolution of migration or enhanced migratory activity in a population, an understanding of the cost-benefit relationship between migration and reproduction is of paramount importance.

A growing body of theory and empirical evidence suggests that migration can in fact be part of a suite of covarying traits, which also includes enhanced reproduction, that are important for successful colonization (Simberloff, 1981, Parsons, 1982, Palmer, 1985). Lines selected for greater dispersal or flight behavior show correlated genetic responses of decreased development time or increased fecundity (Lavie and Ritte, 1978; Wu, 1981; Palmer, 1985): *Oncopeltus* that have been artificially selected for long wings (a trait correlated with greater flight propensity) show higher early fecundity than unselected controls or those selected for short wings (Palmer, 1985). Prolonged flight itself has been shown to have no deleterious effects on reproduction in some species: among alate *A. fabae* there is variation in the tendency to migrate (Shaw, 1970), but given access to food afterwards, alates flown to exhaustion showed no difference from those allowed only a brief flight (to induce settling) in rate of larviposition, total number of larvae produced, or survival of larvae (Cockbain, 1961). In several species flight actually stimulates reproduction (Johnson, 1958; Highnam and Haskell, 1964; Rygg, 1966; Slansky, 1980). In *Oncopeltus* subjected to tethered flight, for example, age to first oviposition and the inter-clutch interval were decreased and the mean number of eggs produced tended to be greater, though not significantly, compared to unflown bugs (Slansky, 1980). Flight greatly accelerates the rate of oocyte growth in laboratory cultures of the locusts, *Schistocerca gregaria* and *Locusta migratoria* (Highnam and Haskell, 1964). Thus while migration or the possession of wings appears to decrease reproductive potential in some species, in others flight accelerates reproduction, is correlated with greater early reproductive output, or has no observed deleterious effects.

Whether an effect of flight on reproduction is seen, an effect of reproduction on flight is often observed. For example, in many insects, migratory activity is greatest between cuticle hardening and the onset of reproduction, and migratory flight behavior of females diminishes as the ovaries mature; male flight behavior may decline on the same timescale (Johnson, 1963, 1969). The decrease in migratory behavior that is often correlated with development of the reproductive tract in the females has been termed the "oogenesis-flight syndrome" (Johnson, 1969). The extent of this negative relationship varies from the extreme of insects that undergo flight muscle histolysis or dealation associated with ovarian maturation (Chapman, 1956; Johnson, 1957; Chudokova and Bocharova-Messner, 1968; Edwards, 1969; Dingle and Arora, 1973; Anderson, 1973; Davis, 1975; Roff, 1984; Johnson, 1963), to species in which an oogenesis-flight syndrome exists, but females are capable of inter-oviposition flight (Johnson, 1963). Even in the extremes, variation exists; for example, some species that undergo flight muscle histolysis regenerate flight muscles after ovipositing and can make interovipositional flights (Chapman, 1956). Furthermore, although a negative effect of mature ovaries on flight is the rule, in some Lepidoptera (Baker, 1984) and [illegible] [illegible]her groups as well, no oogenesis-flight syndrome occurs, and insects may [illegible] distances primarily as a consequence of directionally oriented movement [illegible] oviposition sites.

The grasshopper, *Melanoplus sanguinipes,* is well known for its periodic mass migrations; individuals from non-swarming populations have also been shown to make long-duration, presumed migratory flights (McAnelly and Rankin, 1986). There is considerable interpopulation variation in the proportions of individuals that engage in such migratory behavior, suggesting that different life history strategies have evolved in different habitats (McAnelly, 1984, 1985; McAnelly and Rankin, 1986). In order to clarify the role of migration in the life history and to examine the reproductive costs of migration in different populations of *M. sanguinipes,* we have examined the relationships between migration, reproduction, and age and the hypothesis that migration decreases the reproductive potential of females.

MATERIALS AND METHODS

Except as noted herein, flight testing and rearing procedures were as described in McAnelly and Rankin (1986): 16:8 photoperiod, rearing temperature of 32°C, relative humidity approximately 30–50% and a diet of 50:50 bran and wheat germ plus fresh lettuce. In the stationary tethered flight assay, a flight duration of 60 min was considered to be indicative of migratory flight (McAnelly and Rankin, 1986). Group-reared grasshoppers were housed in 27,000 cm^3 screen and Plexiglas cages. Individuals reared separately were housed in 525 cm^3 screen and plastic cages. As described previously (McAnelly and Rankin, 1986) the grasshoppers were derived from a relatively non-migratory population near Ft. Collins, Colorado (the CO population), and from an actively migratory population on the San Carlos Apache Indian Reservation in Arizona (the AZ population). In addition, grasshoppers from a site approximately 15 miles northwest of Silver City, New Mexico (32° 50′ N and 108° 20′ W) (the NM population) were also used in this study. This population exhibited a very high degree of migratory activity as determined by tethered flight assay (McAnelly, 1984; McAnelly, 1985). After the initial experiment, described in Results, which used only CO animals, an outbreak of the protozoan parasite *Malameba locustae* occurred in the AZ and CO laboratory lines. To prevent a recurrence of this outbreak after diseased stocks were eliminated, in subsequent experiments animals in these two groups were also fed artificial diet containing the sulfa drug, thipyrimeth, as described by Henry and Oma (1975). CO and AZ grasshoppers also were dissected after each experiment and examined for the presence of *M. locustae* cysts (Henry, 1968). No infected individuals were found in the experiments described in this study. NM animals were never exposed to the infection and showed no evidence of the disease and therefore were not treated with sulfa drugs. Although the efficacy of sulfa drugs when administered as an aqueous spray to the food has been challenged (Hanrahan, 1981), a mixture of the drug in an artificial diet at concentrations of 0.4–6.4% was a very effective means of administration and had no deleterious effects on hormone production, reproduction, or hatching (Henry and Oma, 1975; Tobe and Pratt, 1975). We found that experimental results from disease-free NM animals reared without artificial diet or sulfa drugs were qualitatively similar to those of the treated CO and AZ insects (see below).

To allow identification of individuals throughout the course of the experiments, each grasshopper was labeled within 24 hours after adult eclosion with a number printed in permanent ink on the left forewing.

The degree of reproductive development was determined as weight of the reproductive tract (gonads plus accessory gland structures and, in females, lateral oviducts) relative to total body weight in both sexes and the presence of vitellogenic oocytes in females. AZ and CO insects that had been flight-tested in the field (McAnelly and Rankin, 1986) were fixed in formaldehyde:acetic acid:70% ethanol (5:5:100) for 5–7 days, then refrigerated in 85% ethanol. NM field-tested and all laboratory insects were fixed in 80% ethanol. All dissections were performed in 70% ethanol. The two different methods of sample preservation yielded equivalent estimates of the ratio of reproductive tract weight to body weight (McAnelly, unpub. obs.).

Specific protocols for a given experiment are described with the results for that experiment.

RESULTS

The oogenesis-flight syndrome

The relationship between reproduction and migration was examined in group-reared laboratory CO progeny. Length of flight was recorded for each individual on day of sacrifice and compared with the degree of reproductive development seen in the fixed animal. In this experiment, reproductive tract weight did not exceed 10% of total body weight in any female until 6 days and did not exceed 15% until day 7 (Fig. 1). However, after 6 days, the degree of reproductive development in females became quite variable such that in almost any age group there were females whose reproductive tracts comprised less than 10% of body weight and others in which it

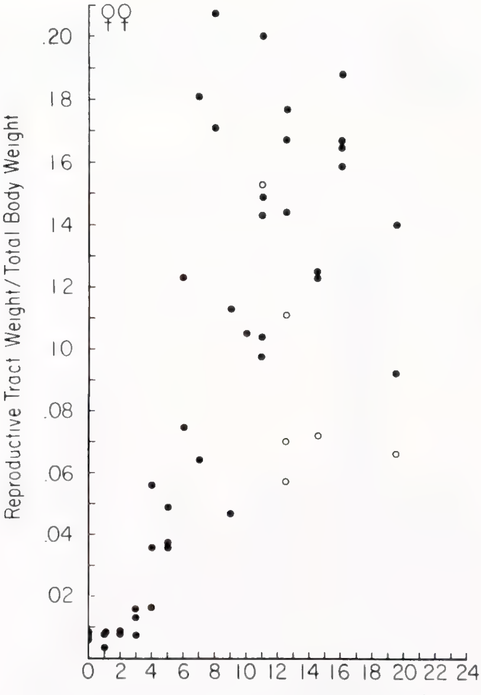

FIGURE 1. The ratio of ovarian weight to body weight increases as a function of age in females. Closed circles represent females with first set of developing oocytes. Open circles represent females in which the condition of ovaries suggested that either oviposition or resorption had occurred.

were greater than 15%. The lower values in older females probably reflected both individual variability in the rate of maturation of the first set of oocytes and the fact that [illegible] females had already oviposited or resorbed oocytes. There was a clear negative relationship between the tendency to make a long flight and the ratio of reproductive tract weight to total body weight in females (Fig. 2). Females in which ovaries comprised at least 15% of the body weight never made long flights and were near oviposition as indicated by the frequent appearance of mature oocytes in the lateral oviducts.

Reproductive tract weight in newly eclosed males was 5.5% of body weight and increased by less than a factor of 2 by day 16 (Fig. 3), compared to an approximately 20-fold increase in females. There was no correlation between male flight behavior and relative reproductive tract weight.

In the initial experiment, floodlights were added for 12 hours during lights-on to increase diurnal temperatures. Grasshoppers preferred positions under the lights and oriented to maximize this exposure. Because the use of floodlights resulted in a range of temperatures (33–36°C) within each cage, not all grasshoppers could be exposed to identical temperatures at all times, and this procedure was not continued in later experiments. Therefore, the exact time course of reproductive development in this experiment may have been somewhat accelerated relative to insects in all subsequent experiments that were reared at 32°C without additional heat sources. Variation in rearing temperature within this range does not alter tendency to engage in long flight (McAnelly, 1984), and the relationship between the state of reproductive development and flight should not be qualitatively affected by differences in rearing temperature (see also below).

Subsequent experiments demonstrated that *M. sanguinipes* females show some tendency for long flight after oviposition. Individual females housed in separate screen cages with a single male were flight tested every other day from age 2 to 28 (3 to 29 in NM grasshoppers) and oviposition cups were checked daily for egg pods. Of those

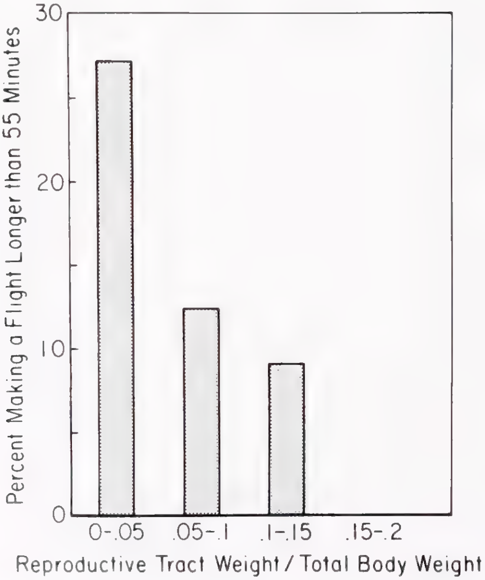

FIGURE 2. The relationship between ovarian development and long flight. Ovarian development was assayed as the ratio of reproductive tract weight to total body weight. Increasing weight of ovaries corresponded to increasing size and vitellogenesis in oocytes. n = 41.

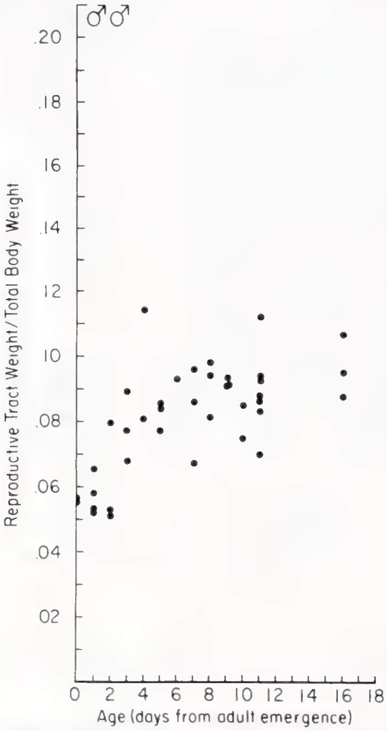

FIGURE 3. Increasing testes weight relative to body weight as a function of age in males.

that made at least one long flight prior to first oviposition, 12.5% (n = 8) of the CO, 35.7% (n = 14) of the AZ, and 20% (n = 25) of the NM females made a long flight after ovipositing. Inter-reproductive flights occurred as early as 24 hours after egg-laying, but it was not possible from this study to pinpoint the minimum interval between oviposition and flight. An example of a sequence of pre- and inter-oviposition flight in an AZ female is illustrated in Figure 4. Long flights by both males and females within 2 or 3 hours of mating were also observed; shorter intervals may be possible.

The relationship between flight and age

The flight behavior of individuals from the CO, AZ, and NM laboratory populations was followed over the first 28–29 days of adult life to examine the relationship between flight and age. By two days after eclosion, the animals were usually no longer soft to the touch, could tolerate the tethering process, and flew readily in the assay. Although this might be considered a brief teneral period relative to *Schistocerca* and *Locusta* (Johnson, 1969), the African grasshopper *Oedaleus senegalensis* has been observed to migrate as early as two days post-eclosion (Riley and Reynolds, 1983).

In all three populations, both males and females showed a decline in flight behavior with age when tested every other day from age 2 through 28 days (day 3 through 29 in NM animals) (Fig. 5). Except for AZ males, this decline is significant for all groups ($P \geq 0.05$) when the proportion of long flights made by each individual during the first two weeks is compared with the performance of that individual during the second two weeks. Small sample size in the AZ male group may account for the lack of

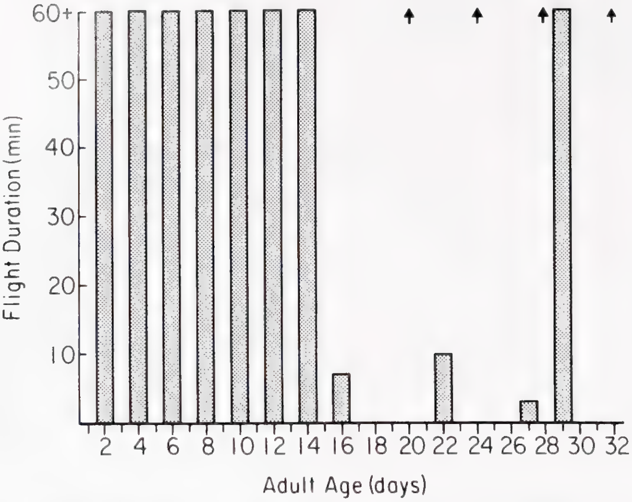

FIGURE 4. Relationship between long flight and oviposition in an AZ female. Shaded bars represent flight duration for each day tested. Arrows indicate dates of oviposition. This individual laid 6 more egg pods after the last oviposition indicated on this figure at day 32.

statistical significance in the decline in numbers of long flights made by individuals, for the trend in this group is similar to that in the other groups. Since each individual was tested every two days, the observed decline in flight behavior with age might have been due to a deleterious effect of repeated flight testing on long flight behavior. Thus NM insects flown every other day from age 2 through 28 were compared with a group flown only twice, once on day 2 and again on day 14. Since these grasshoppers showed no significant difference in flight performance with age when compared to insects tested 7 times, we conclude that this effect is not due to repeated flight testing (Table I).

Under the conditions of this study, the average age at first oviposition for each population ranged from day 21 to day 23, with the earliest observed oviposition at day 15. Thus these results, in conjunction with those discussed above, support the

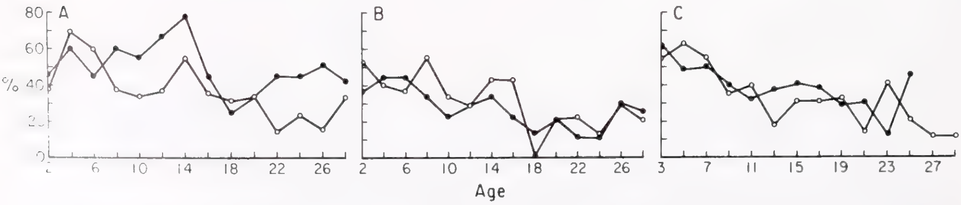

FIGURE 5. Decline in the percent of individually reared grasshoppers making long flights as a function of age. Vertical axis represents the proportion of animals that made 60 minute flights at a given age (in days on x axis). Closed circles represent males and open circles females. A. AZ grasshoppers. n's range from 11 (day 2) to 7 (day 28) in males and from 16 (day 2) to 9 (day 28) in females. Males: $P < 0.145$. Females: $P = 0.029$. [Sign Test comparison of the proportion of long flights made by each individual during days 1–14 with that made during days 15–28 (29 in NM).] B. CO grasshoppers. n's range from 9 (day 2) to 8 (day 28) in males and from 21 (day 2) to 5 (day 28) in females. Males: $P = 0.008$. Females: $P = 0.031$. (Sign Test as above.) C. NM grasshoppers. n's for females ranged from 24 (day 3) to 9 (day 29) and for males from 31 (day 3) to 11 (day 25). Males: $P = 0.048$. Females: $P = 0.011$. (Sign Test as above.)

TABLE I

Comparison of the number of 60 min flights made (on days 2 and 14) by grasshoppers flight tested every second day from day 2 after adult emergence until day 14 with those flown only on day 2 and on day 14

A. Males

	Tested every other day	Tested twice
No change between day 2 and day 14	16	12
Day 2 > day 14	2	3
Day 14 > day 2	0	2

$\chi^2 = 2.65$, $P > 0.10$.
No significant difference between males flown every other day and those flown only twice.

B. Females

	Tested every other day	Tested twice
No change between day 2 and 14	10	7
Day 2 > day 14	2	1
Day 14 > day 2	3	4

$\chi^2 = 0.675$, $P > 0.250$.
No significant difference between females flown every other day and those flown only twice.

conclusion that the decrease over time in the flight behavior of females is correlated with increasing ovarian development. Physiological changes other than reproductive development are associated with aging and also could be involved with flight performance.

The relationship between flight and reproduction in field insects

In all populations, field females with more than 15% of their body weight committed to reproductive development did not make long flights (Fig. 6). If more of the Colorado

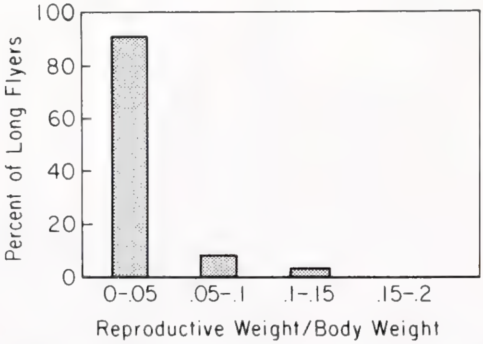

FIGURE 6. The relative degree of reproductive tract development among only those field flight tested females that flew at least 60 min. Data for all 3 populations, CO, AZ, and NM, for all years are combined. Sample size was n = 43.

females were gravid, this could have accounted for a lower level of flight in the CO population (McAnelly and Rankin, 1986), but this was not the case. There were marked differences in mean ovarian development between the AZ and CO 1981 populations, but in the wrong direction to account for the observed differences in migratory behavior by way of an oogenesis-flight syndrome. The more migratory AZ insects in 1981 showed greater average ovarian development than the relatively sedentary CO 1981 females (Fig. 7). Thus, there were proportionately more, not fewer, gravid females in the AZ population, but relatively more of the non-gravid females were migrants than the non-gravid CO females.

The effect of repeated flight testing on oviposition

To determine whether migratory flight exacts a cost in terms of the individual's reproductive output, we examined the age at first oviposition and egg pod production as a function of frequency of flight testing. Individually reared CO and AZ insects were each divided into two groups; one was flight tested every other day from 2 days after adult emergence to day 28 and the other only twice, once on day 2 and once on day 14. To control for the variation in the migratory tendency of individuals (McAnelly, 1985; McAnelly and Rankin, 1986), after the initial flight test on day 2, equal numbers of nonfliers (those that flew less than 60 min) and fliers (those that flew 60 min) were placed in each of the two groups. Thus both groups initially contained approximately equal numbers of "migrants" and "non-migrants," as determined by the initial test. Greater frequency of flight testing in the CO and AZ grasshoppers resulted in higher total flight duration over the lifetime of the animals such that grasshoppers that were tested repeatedly made 60-min flights 4.43 to 5.58 times more frequently than those tested only twice (Table II). NM grasshoppers were divided into a group of grasshoppers flown every other day from day 3 to day 29 and one in which individuals were never flown. In all groups, oviposition containers were checked daily for egg pods.

Performance of long duration flight did not decrease reproductive output. In all three laboratory-reared lines there was a trend, which was significant at the $P < 0.01$ level for the NM grasshoppers, toward decreasing, rather than increasing, age at first oviposition in insects that were flight tested repeatedly (Fig. 8A). There were no significant differences between treatments or between populations in numbers of egg

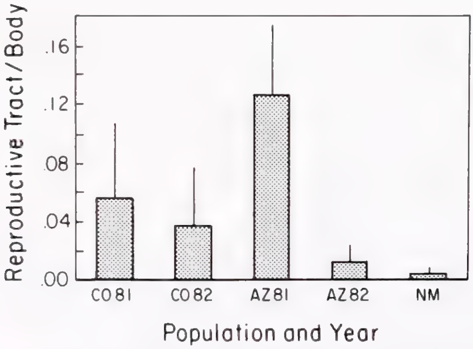

FIGURE 7. The relative degree of ovarian development in field-collected CO, AZ, and NM females calculated as the weight of reproductive tract (ovaries, accessory glands, and lateral oviducts) divided by the total body weight. Values given are means and standard deviations. Sample sizes were n = 33 (CO81), 28 (CO82), 15 (AZ81), 88 (AZ82), 27 (NM).

TABLE II

Comparison of total lifetime flight duration of grasshoppers subjected to different numbers of flight tests

Population	CO		AZ		NM	
Number of flight tests	2×	14×	2×	14×	0×	14×
Total average lifetime flight duration (min.)	55.6	310	89.7	397.4	0	389.4

Within the group labeled 14× some individuals were actually tested only 10–13 times.

pods produced (Fig. 8B). However, the mean number of egg pods laid by CO females tested repeatedly tended to be less than that for CO females tested only twice, and the NM females tested repeatedly laid slightly more pods than those never allowed to fly.

Oviposition in flown and unflown females

Female NM grasshoppers at least 14 days old and that had not been previously flight tested were used to further examine the effect of flight on oviposition. The exact

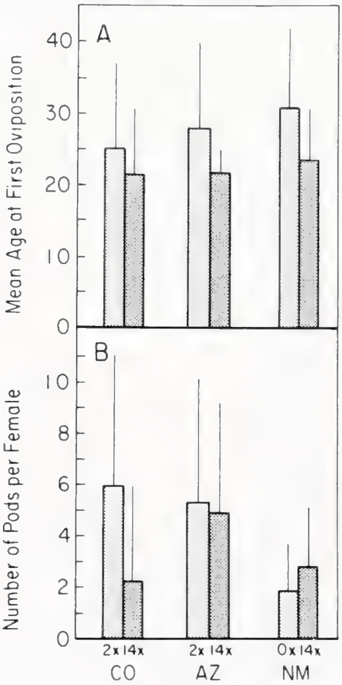

FIGURE 8. A. The effect of flight on age at first oviposition. Comparison of the average age at first oviposition in insects flown every other day after adult eclosion up to 14 times *versus*, for AZ and CO insects, those flown only twice and, for NM insects, those never tested. Data are shown as average + 1 S.D. t = 2.65, df = 28, $P < .01$ for NM grasshoppers. Sample sizes were (for 2× or 0× flights) 5 (CO), 8 (AZ), 23 (NM) and (for 14× flights) 6 (CO), 7 (AZ), and 21 (NM). NS for AZ and CO insects. B. The effect of flight on number of egg pods produced per female as in 8A. Grasshoppers that never oviposited were included in the analysis of the number of pods per female, but not in calculating the age to first oviposition. Data are shown as average + 1 S.D. Sample sizes were (for 2× or 0× flights) 7 (CO), 10 (AZ), 15 (NM) and (for 14× flights) 9 (CO), 8 (AZ), and 15 (NM). NS for any population.

age of these group-reared individuals was not determined. However all had eclosed at approximately the same time and were randomly distributed between control and experimental groups such that the age ranges in each group were equivalent.

Flight tested grasshoppers were given only one trial during which they were allowed to fly without interruption up to 9 h 48 min, with the stipulation that insects that flew less than 1 hour were removed from the experiment. The minimum flight duration, excluding the 2 females that flew less than 60 minutes, was 2 h 45 min with a maximum of 9 h 48 min and mean of 8 h 12 min. Unflown control females were treated in two ways. In the first control, 10 female grasshoppers were tethered and suspended under floodlights but were not exposed to wind stimulation (Parker *et al.*, 1955; Riegert, 1962; McAnelly and Rankin, 1986), and therefore did not fly. The other control consisted of 2 groups of 10 and 20 females, respectively, neither of which had been handled other than during unavoidable routine feeding and cleaning procedures. After flight testing, flown insects and tethered controls were returned to their cages and allowed to pair randomly with an equal number of unflown males. Reproductive output was scored as the number of egg pods laid in each group. No oviposition had been observed in experimental or control groups prior to flight testing. NM females that made a long flight laid significantly more egg pods per female over the subsequent 23-day observation period than those never allowed to fly (Fig. 9). The number of egg pods produced by tethered, heated controls did not differ significantly from controls not handled in any manner.

DISCUSSION

The hypothesis that migratory flight behavior exacts a reproductive cost in *M. sanguinipes* has not been supported by this study. Instead, flight strongly stimulated

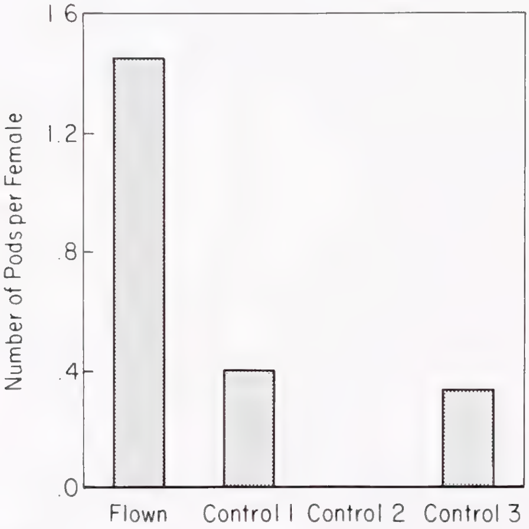

FIGURE 9. Comparison of oviposition in NM females that flew >2 h 45 min in one trial with those never allowed to fly. Control 1 (10 females) consisted of females that were tethered and suspended under floodlights but not flown. Controls 2 (10 females) and 3 (20 females) were not handled in any manner except for routine feeding and cleaning. χ^2 of the total number of egg pods (not the number of pods/female) produced by each group = 29.13, df = 3, $P < 0.001$.

oviposition and decreased the age at first oviposition in NM females, tended to decrease age at first oviposition in CO and AZ females, and, with the possible exception of the CO insects, greater frequency of long duration flight did not decrease production of egg pods. There are several other components of reproductive fitness to be considered in a complete estimation of the cost of migration to reproductive fitness, such as total development time, the probability of survival for animals that stay *versus* those that leave, the total number of eggs, rather than pods, produced over the lifespan of the female, hatching success and the reproductive success of offspring, and whether there are trade-offs between early and later reproduction in migrants and non-migrants. In addition, the possibility that these populations vary in the response of reproductive output to flight (with the relatively sedentary CO population perhaps displaying decreased rather than increased output) requires further investigation. Nonetheless, the evidence from this study suggests that flight is a powerful oviposition stimulus in at least one population of *M. sanguinipes.*

Given that flight has been reported to accelerate reproduction in other species (Johnson, 1958; Highnam and Haskall, 1964; Rygg, 1966; Slansky, 1980) or to be necessary before oviposition can begin (Johnson, 1958; Kennedy and Booth, 1963; Williams, 1958), it may be that flight activity in migrant insects can function as a mechanism to coordinate termination of diapause (see also below) or immediate onset of oviposition with arrival in a suitable habitat. Such a mechanism would greatly facilitate rapid exploitation of new habitats and may be an important component of the life history strategies of many insect migrants.

In the earlier stages of oogenesis, development of ovaries and migration are not mutually exclusive even in many species that display an oogenesis-flight syndrome. Although fully gravid *M. sanguinipes* females (with ovaries ≥ 15% of total body weight) do not make long flights, females with vitellogenic oocytes and with more than 10% of their body weight devoted to ovarian development are still capable of long-duration flight. Similarly, in the species *Oncopeltus fasciatus, Hippodamia convergens,* and *Danaus plexippus,* while females with fully mature oocytes make few if any long flights, migration and oogenesis in these species may proceed simultaneously. On the return spring migrations, *Hippodamia* and *Danaus* migrants leave their overwintering sites, mated and with rapidly developing oocytes. Indeed *Hippodamia* collected at the winter hibernacula appear to require a period of flight prior to oviposition (Williams, 1958). The alate morphs of some species of aphids also delay larviposition until after a period of flight, even if embryos are already well-developed (Johnson, 1958; Kennedy and Booth, 1963).

Inter-ovipositional flights and pre-migratory mating have important implications for migrant life history strategies. Particularly in relatively long-lived species, a female capable of resuming migratory behavior after ovipositing could spread her reproductive risk by placing later pods in different habitat patches or could reduce intraspecific competition among offspring by dispersing them in space.

The ability to make long flights after egg laying has begun can also be a facultative response to declining environmental quality which might allow the female to prolong the reproductive portion of her life. Many species will resume migratory activity after oviposition if they are subjected to starvation or poor quality food (Dingle, 1968, 1972; Rankin and Riddiford, 1977; see Rankin and Singer, 1984).

Although further field work will be necessary to document how frequently and under what conditions *M. sanguinipes* that have already oviposited migrate to new sites, swarming grasshoppers of this species (Parker *et al.,* 1955), as well as some locusts (Johnson, 1963) have been observed to make inter-oviposition flights in the field. The ability of females in the field to migrate after ovipositing could depend on a number of variables such as the availability of food resources or the timing of appropriate weather conditions suitable for flight (see Parker *et al.*, 1955). However, even if such animals are somewhat exceptional, they may represent an important alternate behavioral strategy for reproduction in unpredictable environments.

The widespread success of *M. sanguinipes* as a colonist and consequently as an agricultural pest is likely due to a number of factors: (1) It is polyphagous and capable of thriving in a diversity of habitats (Gurney and Brooks, 1959; Pfadt, 1949). (2) It has a high intrinsic rate of increase (Pfadt and Smith, 1972). (3) Long-duration flight accelerates reproductive development and in some populations may enhance total reproductive output. (4) Most long flight is prereproductive. This, coupled with the acceleration of reproductive development by flight, will result in emigrants arriving at a new site at the peak of their reproductive potential. (5) Inter-reproductive flights in this species can also allow the female to exploit widely separated habitat patches.

This suite of adaptations is similar to those predicted to occur in species that have experienced strong selection pressures for colonization (Mayr, 1965; Dingle, 1972; Safriel and Ritte, 1980). Furthermore, the interpopulation differences in migratory behavior in *M. sanguinipes* have a genetic basis (McAnelly 1984, 1985; McAnelly and Rankin, in prep.) The results reported here thus suggest that reproductive costs have been minimized in populations subjected to strong natural selection for migratory behavior and colonization. Migratory flight may exact a reproductive cost in species or populations that have not experienced such strong selection pressures for movement, such as the CO *M. sanguinipes* population, or those that migrate facultatively in response to currently declining environmental conditions (see references cited in introduction). Comparison of changes in reproductive output in response to long duration flight between migratory and non-migratory *M. sanguinipes* populations will form the basis for further work on this question.

ACKNOWLEDGMENTS

Kristina Tiebel and Robin Perkins provided assistance with rearing animals. Margaret Saks, Harold Zakon, Mike Singer, and Darrell Moore provided helpful criticisms of an earlier version of this paper. This work was supported by NSF grant PCM 8110568 to M. A. Rankin and by University of Texas graduate research awards to Lynne McAnelly.

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THE VISUAL SYSTEM OF THE GIANT CLAM *TRIDACNA:* BEHAVIORAL ADAPTATIONS

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ABSTRACT

Giant clams in the genus *Tridacna* exhibit a number of visual behaviors adaptated to the hypertrophied siphonal lobes and the symbiotic zooxanthellae. The visual system consists of many eyes located along the margin of the siphon. Shadows without abrupt changes in intensity or movement elicit slow retractions of corresponding siphon regions. A similar response also occurs in isolated tissues and appears to rely on peripheral synaptic interactions. Otherwise, a shadow response, or a sight reaction to distant movements, triggers a synchronous rapid retraction of the entire siphon along with adduction of the valves. These abrupt responses are centrally coordinated and have a startling effect on would-be predators due to the water jetted from the exhalent aperture and the visual effect of movement by the large, brightly colored siphon. Animals become habituated to repetitive shadows but remain sensitive to stimuli of different spatial or temporal characteristics, thus illustrating the existence of complex central integrative mechanisms. A behavioral reflex to a light stimulus is also described whereby the surface of the mantle is directed toward the light. This 'orientation response' may represent a phototropic behavior which maximizes the absorption of light by the algal symbionts.

INTRODUCTION

Giant clams of the genus *Tridacna* are prominent members of tropical Indo-Pacific coral reef communities. Five species with overlapping ranges are widely distributed throughout the region (Rosewater, 1965). As the largest representatives of the bivalve molluscs, they exhibit a number of specialized adaptations and these are directly reflected by their habitat preference for relatively shallow (maximum depth, 20 m; Hardy and Hardy, 1969), clear, tropical seas. Most importantly, *Tridacna* hosts the photosynthetic endosymbiotic zooxanthella *Symbiodinium* (Freudenthal, 1962), and has come to rely on the algal photosynthates as a source of metabolic carbon (Trench *et al.,* 1981). In effect, *Tridacna* farms the zooxanthellae in large numbers in the tissues of its hypertrophied mantle siphon, the lobes of which are deployed past the shell margin and intentionally exposed to the intense solar radiation of the tropical marine environment.

In this 'farming' posture, the siphonal lobes are unprotected by the shell and, as observed by Stasek (1965), are subject to predation by reef fish. In his paper, Stasek describes generally the protective behavioral adaptations of *Tridacna,* including powerful spurts of seawater from the siphon apertures which can be directed toward the source of a tactile stimulus. A similar spurting reflex is mediated visually, presumably by the mantle eyes (Stasek, 1966), although a directional component has yet to be

Received 22 May 1985; accepted 6 March 1986.

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demonstrated for this stimulus modality. Visually mediated behaviors include the shadow response, a withdrawal reflex common to most bivalves (Land, 1968), and the "sight reaction" in which reflex withdrawals are produced by moving objects even though their shadows fall to one side of the animal (Stasek, 1965). The shadow response of *Tridacna* is well known to the casual observer of the reef environment, including collectors and sport divers (Kawaguti and Mabuchi, 1969), and brief descriptions or anecdotes have been reported in the *Tridacna* literature by other authors (McMichael, 1974; Yonge, 1980; Fankboner, 1981).

The repertoire of *Tridacna* visual responses is further described in this report. Direct recordings of shell and mantle movements are used to analyze these behaviors. A sequence of increasingly more complex responses is presented ranging from local retractions, which also occur in isolated pieces of the mantle, to coordinated retractions of the entire siphon in concert with partial valve adductions. In addition, a novel phototrophic behavior is described which appears to facilitate maximal light absorption by the mantle, and which illustrates the capability for spatial resolution by the visual system.

MATERIALS AND METHODS

Several species of *Tridacna* were used in this study. Specimens of *T. maxima, T. squamosa,* and *T. derasa* were collected at Davies Reef, a platform reef on the Great Barrier Reef in N. Queensland off the coast from Townsville, Australia. The burrowing tridacnid *T. crocea* was collected from fringing reefs at the Palm Islands north of Townsville. Field observations were made with the aid of SCUBA on Davies Reef, and on Harold's Cay (Flinders Group) in the Coral Sea. Laboratory facilities were provided by the Australian Institute of Marine Science (AIMS) at Cape Ferguson and aboard the RV *Sirius* (February, 1981) and RV *Lady Basten* (June, 1981). Additional experiments were performed on small animals maintained in sunlit marine aquaria in Canberra.

Behavioral recordings

Movements of the shell and mantle of *Tridacna* were recorded by means of dual-arm strain gauge transducers and bridge circuits constructed in the laboratory and powered by 6 V batteries. In one series of experiments, shell movements alone were recorded from a large *T. derasa* (shell length, 45 cm) maintained in an outdoor running seawater table at the AIMS laboratory. The animal was covered by 2 cm of water at the midpoint of the siphon. A transducer was mounted on the side of the tank and attached by a small hook and rubber band to one of the valves. Measurements of valve gape were correlated with pen excursions on the chart recorder and found to be reasonably linear over the range of movements recorded (Fig. 1). Experimental results were obtained around midday on clear sunny days; during the course of an experiment radiant flux varied from 0.75 to 1.62×10^3 Einsteins/m^2/s, as measured with a Quantum Radiometer Photometer (Li-Cor). Shadows from a hand-held wand (a 7 × 14 cm rectangular piece of opaque plastic attached to the end of a 1 m dowel rod) were presented by passing the wand transversely across the posterior region of the siphon at the level of the inhalent aperture. The long axis of the plastic wand was parallel to the ventral margin of the shell and the characteristics of the shadow were maintained as constant as possible in terms of the location, velocity, and height (20 cm) of the stimulus. A photocell was positioned adjacent to the leading valve to monitor the onset of the shadow, and a barrier was erected to visually screen the experimenter from the animal's view and protect equipment from the resulting jets of seawater.

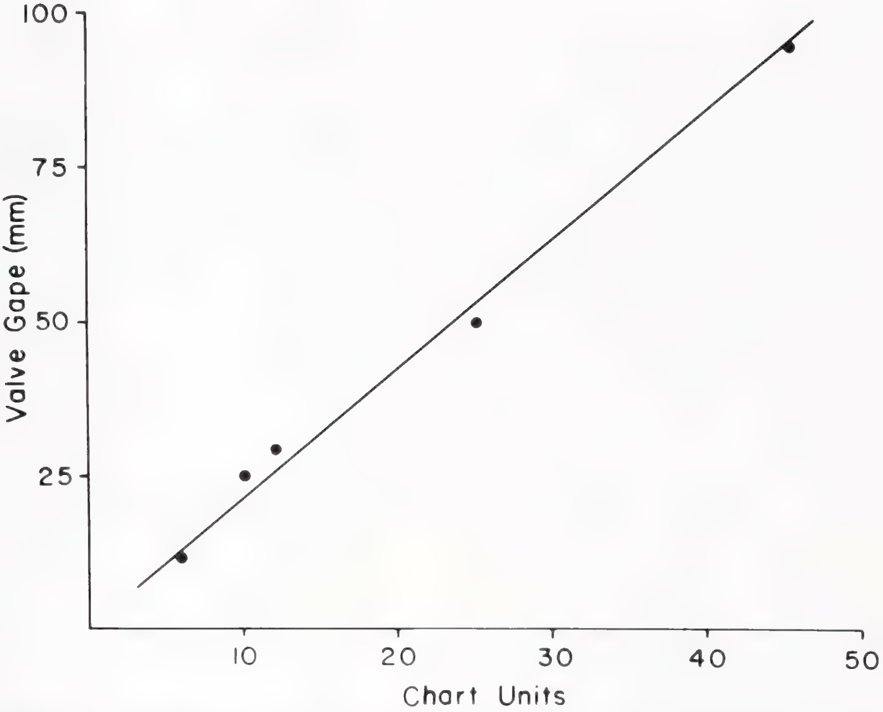

FIGURE 1. Calibration of strain gauge. Direct measurements of valve gape at the midpoint of the siphon in *Tridacna derasa* are plotted relative to pen movements. For a moderately large animal, with a shell length of 45 cm, maximum gape was 15 cm and the minimum gape, after considerable mechanical prodding, was 1.2 cm.

In other experiments, both shell and siphon movements were recorded from smaller *T. maxima, T. squamosa,* and *T. crocea* (13–18 cm) which were clamped by their left valve and suspended in a tank of aerated seawater in the laboratory. With this valve as the fixed reference point, shell movements were recorded from the right valve, as previously described, while siphonal retractions were recorded from the left side. A small wire hook inserted into the center margin of one of the siphon lobes was attached to a second transducer to record siphon movements. Overhead illumination was provided from a xenon arc source fed through a fiber optic light guide positioned 25 cm above the animal. An experimental 'shadow' was produced by closing an electronic shutter located between the light source and light guide.

Histology

A preliminary histological examination of the siphonal eyes was made by removing small pieces of the mantle followed by fixation in 10% formalin. Thick sections of plastic-embedded material were examined and photographed with a Zeiss Photomicroscope II.

RESULTS

General morphology of the siphon and eyes

The upper surface of *Tridacna* is composed of a series of deeply-curved siphonal lobes (Fig. 2) and an interconnecting mantle shelf. Each lobe is the greatly hypertrophied

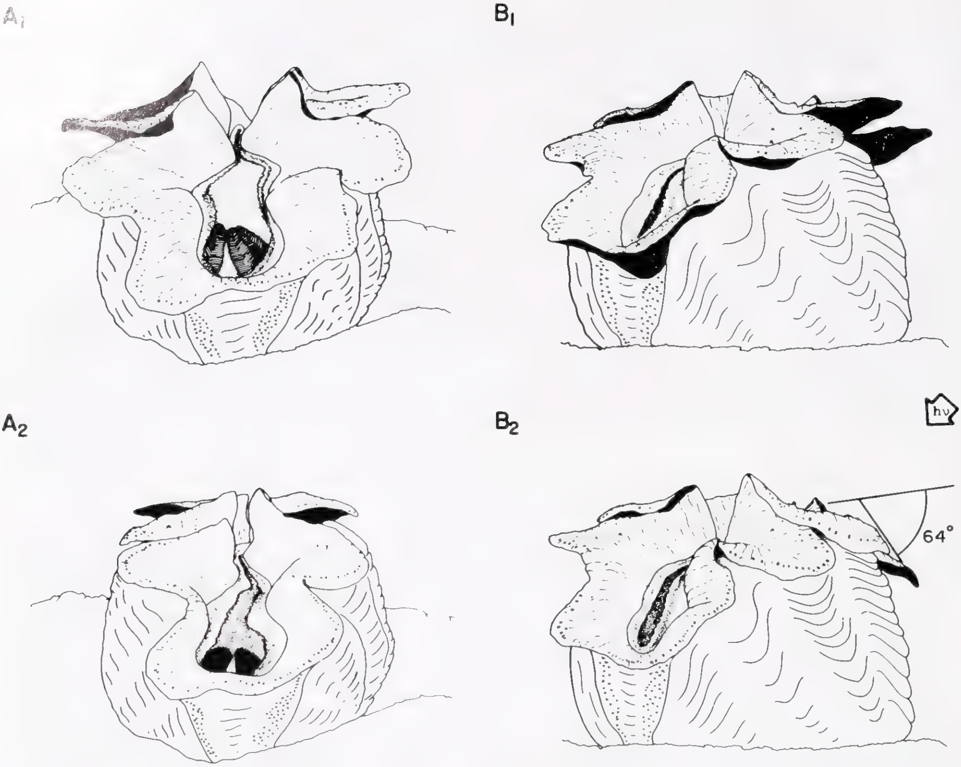

FIGURE 2. Light-mediated siphon responses in *Tridacna maxima* (shell length, 10.8 cm) maintained in a laboratory aquarium, and viewed from the posterior region showing the inhalent aperture and gills. Drawings were made by tracing images projected from 35 mm color slides. The top figures (A_1, B_1) illustrate the fully extended posture of the siphon exhibited when the animal is exposed to direct sunlight. A symmetrical retraction of the siphon lobes typical of either the shadow response or sight reaction is illustrated in A_2. This is a synchronized response and is frequently coupled with valve adduction, although not in this instance, and with partial gill retraction. The orientation response is illustrated in B_2. An additional light source, positioned at an angle of approximately 45° from the right side of the animal, results in a lowering of the siphonal lobes which face the light. In this instance, the lateral surface of the siphon was lowered 64°, as indicated by an arc drawn relative to the position of the siphon in B_1. No other movements are associated with this response.

extension of the inner marginal fold of the mantle (Yonge, 1936) and these are extended laterally past the edge of the shell. The fullest extension of the mantle is observed in animals that occur below optimum depths, (R. D. Braley, pers. comm.). This posture is illustrated in Figure $2A_1$ for a medium-sized *T. maxima.* In large specimens of *T. maxima,* and in the larger species (*T. derasa* and *T. gigas*), extension of the mantle past the shell margin does not increase in proportion to the size of the animal. Rather, the increase in upper surface area is associated with a deeper scalloping of the valves forming shell teeth, and the concomitant widening of the siphonal lobes. The interconnecting mantle shelf also assumes a greater proportion of the upper surface area in large animals.

The eyes, numbering in the thousands in large animals (Fankboner, 1981), are located primarily around the margin of the siphon (Fig. 2), and are readily distinguished in most animals by a concentrated ring of algal cells. A few additional eyes are located on raised tubercles scattered over the mantle surface, although generally not on the interconnecting shelf region. The structure of the eye (see also Stasek, 1966) is that of an oblong capsule (Fig. 3A). The wall of the capsule is slightly constricted beneath the surface of the eye resulting in a fairly wide aperture. A layer of retinal cells lines the lower two-thirds of the capsule out of which an optic nerve extends into the adjacent tissues. Lens cells occupy the remaining interior portion of the capsule and are covered by a thin layer of epithelial cells at the surface. Each of the marginal eyes, whose orientation is indicated by the position of the lens and the contour of the mantle surface, faces laterally and above the plane of the siphon surface (Fig. 3B). Where the siphon is characterized by distinct, curved lobes, each eye is aligned with the radius of the lobe, a direction indicated relative to the radiating iridescent patterns of the animal as depicted in Figures 2 and 3B.

The varying orientation of numerous eyes effectively constitutes a type of mosaic visual system although considerable overlap occurs in the receptive field of the various eyes. This results in part from their location on adjacent folds of the siphon. In addition, the receptive field of each eye will be wide since the lens, in contact with the retina and having a refractive index presumably close to seawater, is not suitable for focusing and retinal cells extend well up along the sides of the capsule (Fig. 3A).

Shadow response behaviors

Local siphon retractions. The familiar visual response for which *Tridacna* is noted is the synchronous siphon retraction and shell adduction which results in a spout of seawater. A variety of visual stimuli can release this behavior. In contrast, slow local retractions of the siphon also exist. These are elicited only by shadows which cover a limited portion of the mantle such as a single fold, or a region bounded by as few as 3–4 eyes. To elicit this response, and avoid more vigorous siphon retractions, the shadow must approach the mantle edge gradually. The resulting indentation of the mantle in effect draws the siphon away from the shadow and reilluminates the tissue.

Local mantle retractions are the result of slow muscle contractions unlike the sudden movements characteristic of synchronous mantle retractions (*e.g.*, Fig. 4). Local responses may be due solely to peripheral interactions between receptor and effector systems, an hypothesis supported by the fact that similar responses occur in small isolated pieces of the mantle. Indeed, it is difficult to eliminate movements in a piece of mantle tissue containing a single eye. This was apparent while recording electrophysiologically from photoreceptors in the eye (Wilkens, 1984), where movements in response to dimming were a source of continual difficulty.

Synchronous retractions. A variety of synchronized responses are triggered by visual stimuli which involve retractions of the exhalent cone, of several lobes of the siphon, or the entire mantle surface, the latter often in concert with a partial adduction of the valves. An effective stimulus can range from direct shadows to visual stimuli producing the sight reaction. Responses exhibiting the greatest visual sensitivity, that is, to movements at a distance, are found in the cone-shaped exhalent aperture. These take the form of slight retractions, and/or an infolding of the lips. These movements occur independently, but also frequently precede the more vigorous siphon responses.

The extent of the visual response is determined in part by (1) the size and velocity of a moving object, as demonstrated by experimenting with animals in the field or in laboratory tanks, (2) the relative decrease in intensity of a controlled light stimulus, and (3) the behavioral state of the clam, a condition determined in part by the frequency of stimulation. In an undisturbed animal the threshold for a synchronized retraction of most or all of the siphon is low, although valve adductions frequently do not ac-

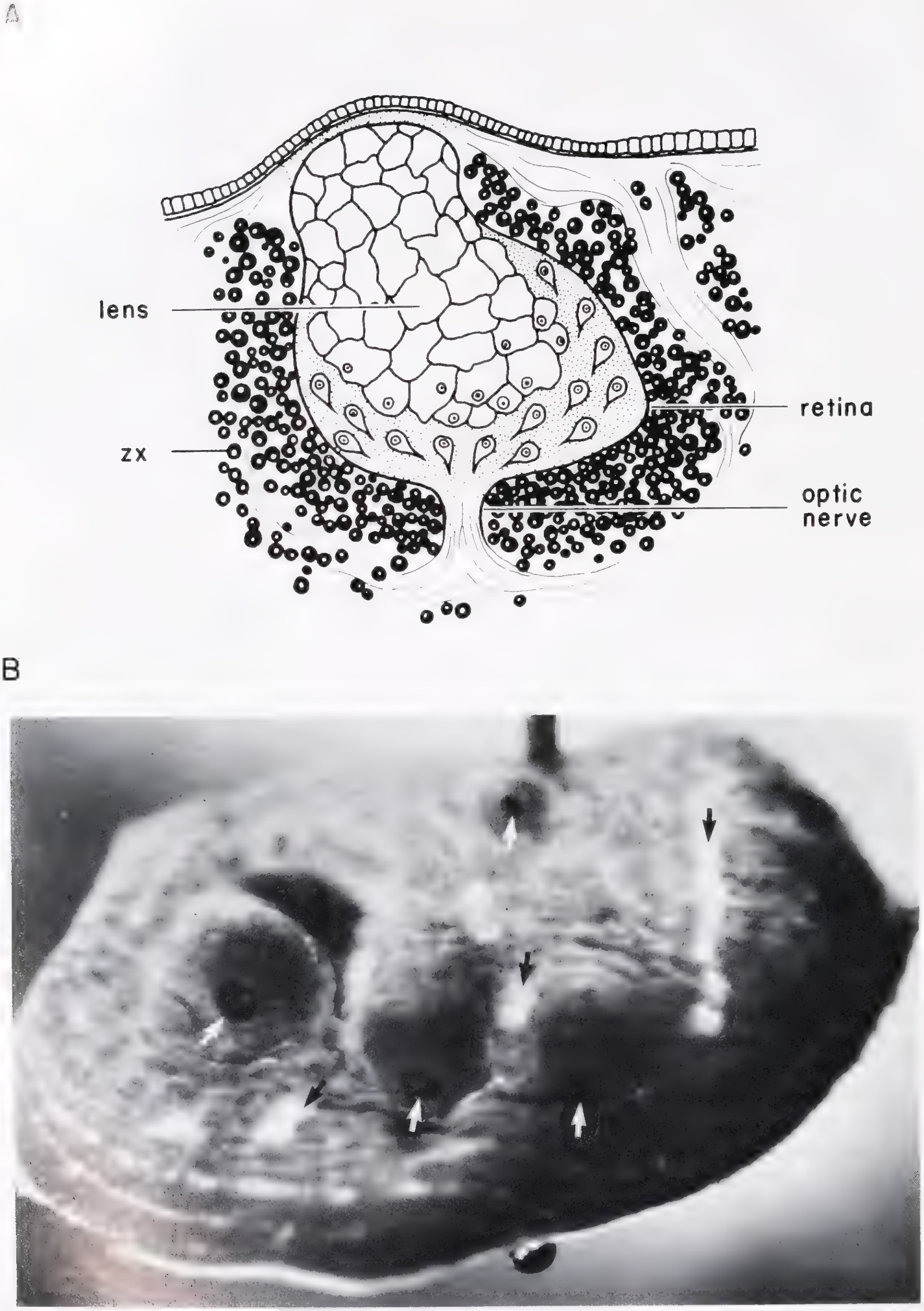

FIGURE 3. The eyes of *Tridacna* illustrated schematically in sagittal section (A, from histological sections), and in a piece of tissue freshly taken from the margin of the siphon in *T. maxima* (B). The morphology of the eye in A corresponds with previous results by Stasek (1966). Photoreceptors are shown with short processes extending toward the optic nerve as observed in anatomical studies with dye-filling of

company this response in the smaller tridacnid species. For example, no shell movement occurred in the visual response illustrated in Figure 2A.

Direct recordings of shadow response movements, including those of both the siphon and shell, are illustrated in Figure 4. A range of visual and spontaneous behaviors is seen. For example, a brief interruption of the overhead illumination may trigger only a partial siphon retraction and no shell movements (A, B). Occasionally, a partial siphon retraction (at arrow in C) will precede a more vigorous retraction and synchronous valve adduction which, in this instance, leads into a sequence of four synchronized siphon/shell movements. The visual response to a shadow, or shadow-like stimulus, therefore ranges from a partial siphon retraction to a sequence of vigorous movements, the latter signifying an elevated state of excitability. Spontaneous activity ranges from frequent slight twitches of the siphon to synchronous, near maximal movements of the siphon and shell (C) which occasionally obscure the visual response (Fig. 4A, at second shadow).

By contrast, a mechanical stimulus to the siphon nearly always triggers a rapid shell and siphon response (Fig. 4B). Even light brushing of the mantle produces valve adductions greater than those elicited by visual stimuli (*e.g.*, last trace, Fig. 5 where the amplitude is 40% greater). Records of valve adduction in the large clam at fast chart speeds (not shown) also show that the maximum rate of shell closure is more rapid (0.370 m/s) following a mechanical stimulus to the siphon than it is for shadow responses (0.275 m/s).

Muscle activity. An analysis of siphon and shell activity reveals that most movements are characteristically the result of sudden contractions which produce steeply rising recorded traces (*e.g.*, Fig. 4–6). The siphonal muscles along the margin of the outer folds are an exception in producing local retractions with a more gradual onset. The more distinctly organized bands of siphon retractor muscles which attach to the shell, and the single adductor, which is composed primarily of "quick" fibers (Yonge, 1980), are responsible for the respective rapid movements of the siphon and shell. The twitch-like contractions of the siphon retractors are present throughout the range of movement amplitudes observed and are likewise characteristic of both reflex responses and spontaneous movements.

Spontaneous siphon retractions occur over a range of amplitudes except in conjunction with spontaneous valve adductions where a full or near-maximum retraction is invariably observed (Fig. 4). Visual responses which elicit shell movements also trigger full retractions of the siphon. The endogenous nature of spontaneous movements is confirmed here by the behavior of animals housed singly in aquaria, and is associated with irrigation of the mantle cavity and periodic removal of feces and pseudofeces (Stasek, 1965).

Whereas both siphon and adductor muscles nearly always exhibit twitch-like contractions, the relaxation phase of these movements is quite variable. The siphon musculature relaxes more rapidly and in many instances reaches half-maximum amplitude prior to the onset of relaxation in the adductor muscle (Fig. 4A). Slower rates of siphon relaxation follow a mechanical stimulus (Fig. 4B), in contrast to those that follow

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the receptor cells (author's unpublished results). Abbr.: zx = zooxanthellae. Three eyes from the marginal row are seen in B, plus a smaller eye located back from the edge of the siphon adjacent to the pin (0.4 mm in diameter) used to secure the tissue. The eyes face outward from the mantle edge; white arrows marking the position of the lenses are oriented parallel to the axis of each eye. Here, the eyes stand out from the surface in a slightly exaggerated fashion due to the partially contracted state of the tissue. Iridophores form radial bands (black arrows) parallel to the orientation of the eyes. A bubble of oxygen (lower edge) arises in response to strong illumination of the zooxanthellae from a microscope lamp.

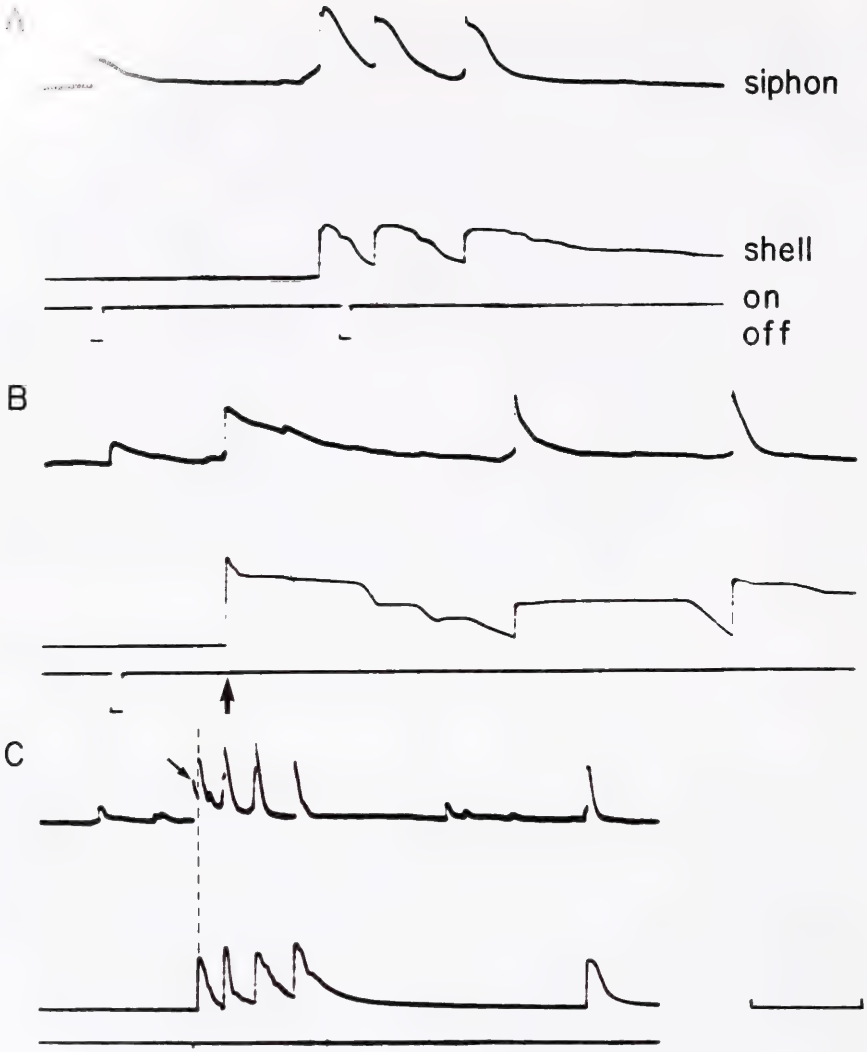


Figure 4. Movements of the siphon and shell in *Tridacna maxima.* (A) A shadow response followed by spontaneous activity. (B) A sustained contracture in response to a mechanical stimulus to the siphon (arrow) follows a shadow response. (C) Spontaneous activity and a sequence of vigorous activity following a shadow stimulus. Movements due to muscle contractions correspond to upward deflections in this and subsequent figures. A fiber-optic light source was located above the animal and its output controlled by an electronic shutter (monitored in the lower trace of each sequence). A vertical dashed line indicates siphon and shell movements in register in C. Time mark indicates 30 s (A, B) and 2.5 min (C).

spontaneous or visually evoked behaviors. A long-lasting retraction of the siphon occurs only when stimulation is sufficiently intense to cause substantial shell closure. The adductor muscle, on the other hand, frequently displays catch-like contractures as inferred from shell movements. The valves often remain stationary following adductions, particularly in response to tactile stimulation (Figs. 4B and 5, last record),

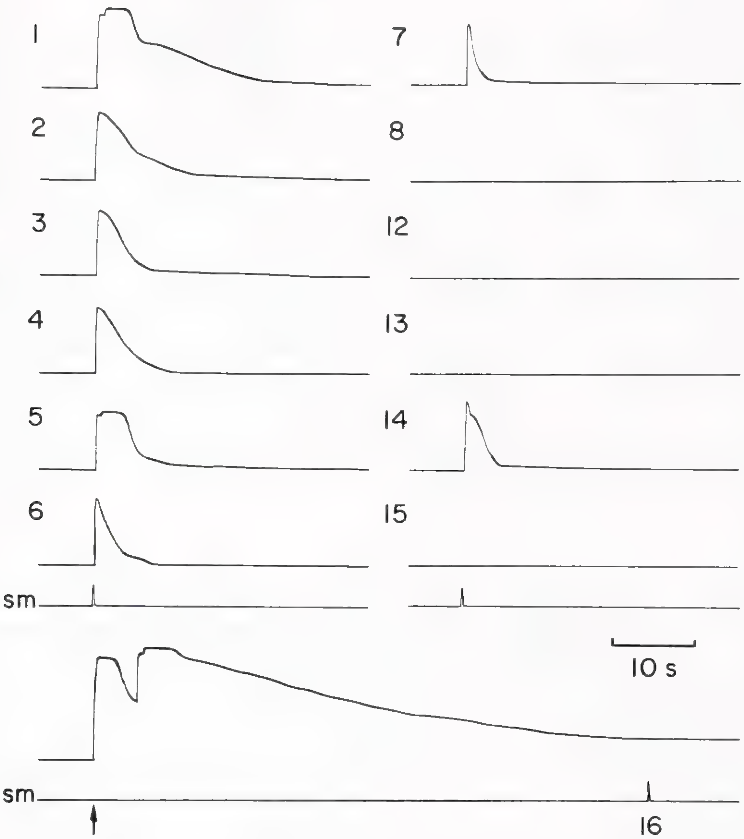
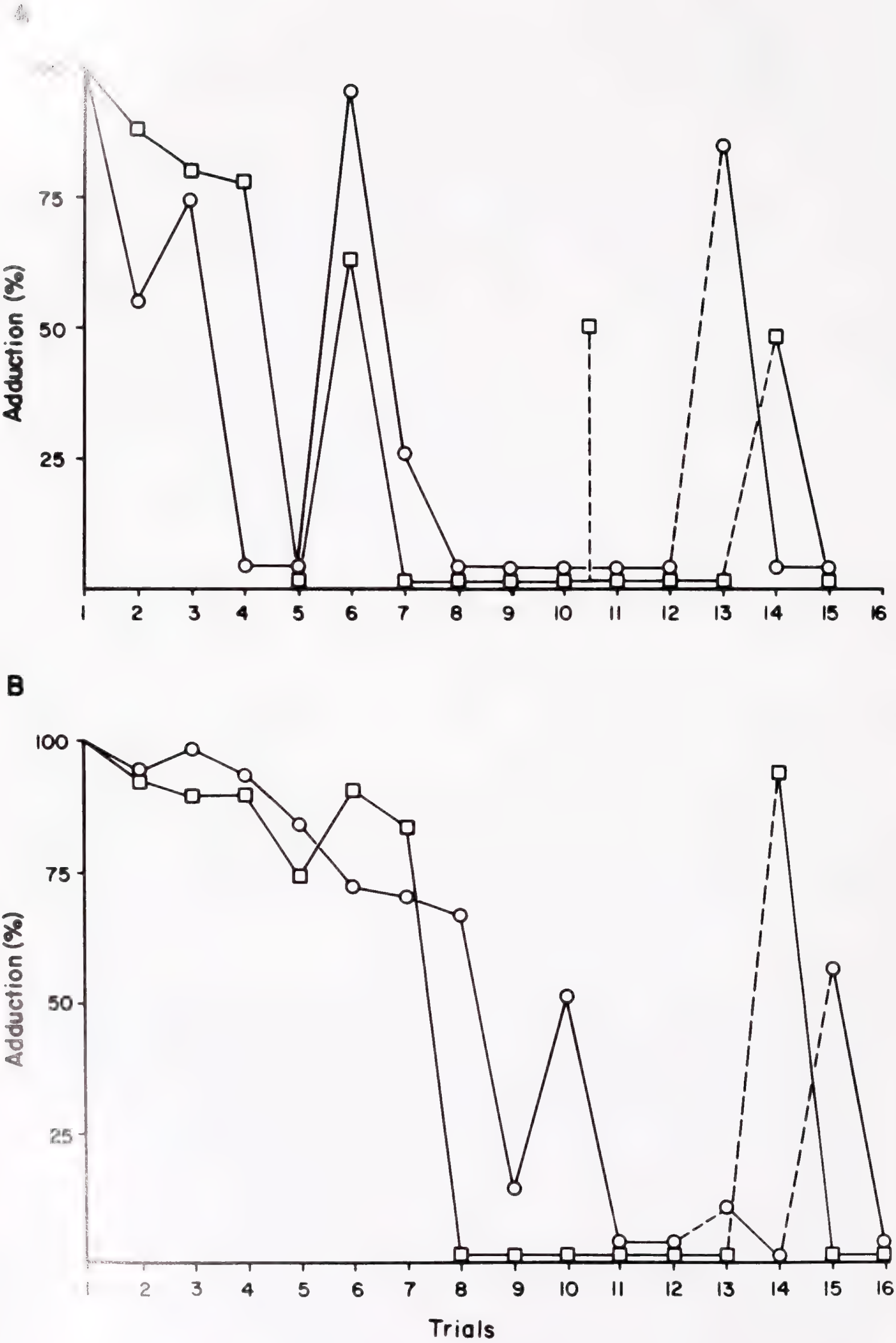


FIGURE 5. Shell movements in *Tridacna derasa.* This animal was maintained in an outdoor tank and received direct sunlight. Shadows were monitored by a photocell clamped near the edge of the shell. Stimulus monitor (SM) traces indicate the onset of shadows and a light tactile brushing of the siphon (arrow) in the bottom record. Traces are numbered according to shadows presented at two-minute intervals. Valve adductions were recorded for seven consecutive stimuli but did not occur in response to six additional shadows at the same location (trials 9–11 were omitted). In trial 14, the position of the shadow was moved to cover a different set of eyes. Trials 15–16 were at the original site.

and relaxation involves plateaus in the catch state as the valves reopen. It should also be noted that all of the valve movements recorded here can be attributed entirely to the adductor muscle, and not the pedal retractors. Through the byssus attachment and the fulcrum effect of the shell umbones, the pedal muscles exert considerable closing force when the clam is attached to its coral substrate (Stasek, 1965). However, in these experiments the byssus of *T. maxima* was attached only to loose gravel in the aquarium, while the larger *T. derasa* no longer possessed a byssus.

Habituation of the visual response. The shadow response of *Tridacna* habituates to repetitive visual stimuli. For example, animals found in shallow water do not respond to the constant flickering of light caused by surface waves (Stasek, 1965). To characterize the behavioral decrement, shell movements of a large *T. derasa* were recorded in response to shadows given at two-minute intervals. An example of an habituation test sequence is presented in Figure 5. The magnitude of valve adduction decreases gradually over successive trials in response to a constant stimulus, that is, to shadows affecting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the same part of the siphon; the shell response eventually habituates completely (*e.g.*, by trial no. 8). Following the habituation of shell movements, however, shadow stimuli continued to elicit siphon retractions that produced jets of seawater, although these also became weak by trial no. 12. Shadow response valve adductions for four habituation test sequences are plotted in Figure 6. The decline of the response was similar for each of the two test sequences (separated by a 30–45 min rest period), although the number of trials eliciting a response varied from six to ten in this series of experiments.

Additional integrative features of the visual system were also apparent during these experiments. For example, the clam habituated to a shadow presented in one location responded vigorously when the eyes from another part of the siphon were shadowed. This effect is illustrated in two instances: (1) after trial stimuli no. 8–10 produced no response (Fig. 6A, curve □), the animal responded vigorously to the passing of a cloud (Fig. 7); (2) in all trial sequences a change in the location of the experimental shadow produced a vigorous valve adduction. A subsequent shadow at the original site was ineffective in each case. A change in duration of the stimulus is also effective in triggering a response (Fig. 6B, curve □, trial no. 14).

Orientation response

In bivalves, only sudden decreases in light intensity have been reported to trigger reflex behaviors—the familiar shadow response. *Tridacna,* however, exhibits a highly reproducible behavior in response to an increase in light intensity. This behavior is described based largely on observations of animals held in laboratory aquaria.

When illuminated under conditions ranging from full sunlight to fluorescent room lighting, an animal with expanded siphonal lobes invariably responds to an additional light stimulus by changing the orientation of its mantle surface relative to the incident light path. For example, light from a flashlight or fiber optic source directed toward one side of the animal will cause siphonal folds facing the light to curve downward. This response is illustrated in Figure 2B by drawings made from photographs taken before and 2 s following the onset of light. Each of the lobes facing the light has been lowered, in this case over an arc of greater than 60° relative to the initial posture (*cf.* Fig. $2B_1$–B_2). Although the entire lobe is affected, the response is most noticeable near the lateral margins where the change in position, up to 83.5° in one instance, results in the upper surface becoming approximately orthogonal to the direction of the additional light stimulus. The lobes on the side opposite the stimulus light do not respond. The directional component of the response can be further demonstrated by changing the position of the light source. In an anterior or posterior position along the midline of the animal, corresponding segments of each of the siphon lobes facing the light on both sides of the animal curve downward. The directionality of this response demonstrates the capability of the visual system for at least coarse spatial resolution.

In a few instances, a light stimulus will trigger a coordinated siphon retraction and partial shell closure instead of the orientation response. This is believed to be due to

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FIGURE 6. Habituation of the valve adduction response to shadows. On successive days, with results plotted separately in A and B, sequences of shadow stimuli were presented at two-minute intervals before (○) and following (□) a recovery period (30 min in A; 45 min in B). Upward broken lines indicate a change in shadow location from the site at which habituation occurred in A (curve □, no. 14; curve ○, no. 13) and B (curve ○, nos. 13, 15): in B (curve □, no. 14) the broken line indicates an increase in shadow duration. The vertical broken line in A indicates the response to a cloud (shown in Fig. 7). The second trial sequence (□) in B corresponds to results shown in Figure 5. All data are from the same animal.

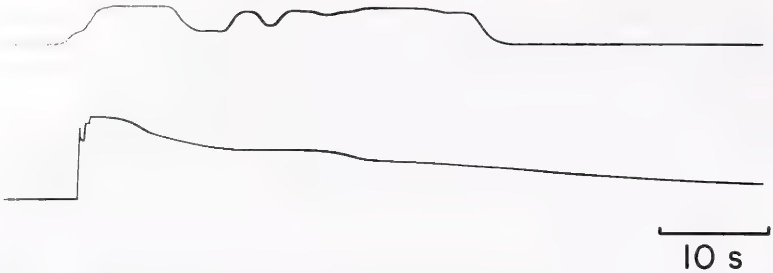

FIGURE 7. Shadow response in *Tridacna derasa* to the passage of a cloud (photocell monitor, upper trace). The change in radiant flux was 0.02×10^3 Einstein/m^2/s; decreased flux is indicated by an upward deflection. Since *Tridacna* responds well to shadows controlled by a shutter, the "cloud response" is presumed to be due to a decrease in light intensity rather than a perception of movement by the cloud.

a difference in the state of excitability of the clam, with the defensive withdrawal response overriding the orientation behavior.

DISCUSSION

Behavioral adaptations

The shadow response. Giant clams are highly specialized bivalves, having evolved as hosts for internal photosynthetic symbionts in parallel with hermatypic reef-building corals. Their principal morphological adaptation is the hypertrophy of the mantle siphon which is positioned uppermost for exposure to light. Visually mediated behaviors are a major component of the protective mechanisms employed by *Tridacna.* Visual responses occur predominantly following a decrease in light intensity, either as the sight reaction or in response to direct shadowing. A characteristic of these behaviors is the twitch-like suddenness of both mantle retractions and shell closure. Since the siphon does not ordinarily retract completely inside the shell, the effectiveness of the response is due in part to a "startle effect" produced by the surge of water from the siphon apertures. This response is more forceably triggered by tactile stimuli, either to avoid or limit the extent of predation. The advantage of the visual response must therefore be to startle would-be predators at a distance, perhaps greater than that served by the water jet. Thus, it is hypothesized that the sudden movements of the large, brightly colored siphon constitute a visual component of the startle behavior. As described here, the rapid nature of siphon retractions and their frequent use independent of valve adduction supports this hypothesis. McMichael (1974) further suggests that the non-cryptic color patterns may serve to warn or confuse potential predators.

These visual behaviors are in sharp contrast to those of the scallop, another of the small number of bivalves with a well-developed visual system. Here, the onset of valve adduction is gradual due to selective activation of the slow, smooth-muscle component of the shell adductor (Wilkens, 1981). In tridacnids, where the mass of the adductor muscle is 85% twitch fibers (Maynard and Burke, 1971), as it is in the scallop, the shadow response includes activation of this fast component. Other bivalves also display sudden, light-mediated adductions (*e.g., Arca,* Braun, 1954; *Spondylus,* pers. obs.). The smooth adductor muscle in *Tridacna* nevertheless appears to be coactivated and is responsible for catch contractures characteristic of both visual and tactile responses. The stepwise relaxations and pre-contraction gaping movements (Fig. 4) suggest that neural mechanisms for the release of catch may be similar to the plasticizing efferent control mechanisms in the scallop (Wilkens, 1981).

The orientation response. Bivalves in general are insensitive to a light-on stimulus with respect to short-term reflexes (Land, 1968), even though in some species (*e.g., Pecten,* Hartline, 1938; *Lima,* Mpitsos, 1973), light-on sensitive cells are represented equally in the pallial nerves with the light-off fibers presumed to be responsible for the shadow response (Land, 1968). The soft-shell clam *Mya* is an exception, reacting to an increase in light intensity by retraction of the siphon, although shadows produce a closure of the siphonal apertures (Wenrich, 1916; Hecht, 1918). The orientation response in the siphonal lobes of *Tridacna* is therefore one of the first examples of a bivalve reflex behavior triggered by an increase in light intensity.

The significance of this response is uncertain. One interpretation is that the siphon movements constitute a positive phototropic response that insures maximum absorption of light by the endosymbiotic algae. Phototropic behaviors also occur in the elysioid gastropods which retain symbiotic chloroplasts in their digestive tract and control light exposure by opening or closing the overlying parapodial flaps (Rahat and Monselise, 1979; Weaver and Clark, 1981). However, it is doubtful that a sudden light stimulus, equivalent to the experimental stimuli used in this study, would occur naturally in the environment of the clam. The reflex nature of the orientation response may therefore be a manifestation of the visual mechanisms which otherwise give rise to continual phototropic adjustments of the siphon. Any effect of light on the orientation of the siphon lobes would also depend on ecological factors such as depth, reef topography, and, perhaps, species. For example, *T. gigas* and *T. derasa* partially covered by coral outcrops orient their mantle surfaces toward available light (R. D. Braley, pers. comm.). Also, when these species are found lying on their side at the base of patch reefs and knolls, phototropic orientation of the siphon is evident.

Insofar as siphon orientation is related to illumination, the degree of siphon extension may also be visually mediated. At night the siphon lobes are partially retracted (Morton, 1978). With light adaptation, however, partial siphon extension develops along with sensitivity to shadows. During the day, the siphon is deployed with the valves widely separated. However, a total consensus on the degree of siphon extension during conditions of maximum insolation is missing. The greatest extension of the siphon in laboratory animals, equivalent to that depicted in Figures $2A_1$ and $2B_1$, occurred only when they were exposed to bright sunlight. Previously, Yonge (1936) noted that the "thickened mantle edges" were widely exposed even when only just covered by water. However, Braley (pers. comm.) has observed that, under the brightest conditions, animals located within 1 m of the reef surface do not extend their siphonal lobes as completely as those found at greater depths. Other invertebrates in the upper sublittoral environment that host zooxanthellae also partially retract their tissues in bright sunlight (Shick and Dykens, 1984; Mangum and Johansen, 1982; Porter, 1980), perhaps as a mechanism for reducing UV exposure (Shick and Dykens, 1984). Partial siphon retraction or a curling down of the lobes in *Tridacna* may also serve to decrease UV exposure. The eyes in *Tridacna* contain UV receptors (Wilkens, 1984), thereby establishing the sensory basis for this behavior.

Physiological mechanisms of visual behaviors

The visual system in *Tridacna* underlies a variety of behaviors including reflex responses to light and shadows. These behaviors raise interesting questions in relation to the physiological properties of the retinal cells. Unlike the scallop, which has photoreceptors which generate spikes in response to shadows (hyperpolarizing) as well as light (depolarizing) (McReynolds and Gorman, 1970), *Tridacna* eyes contain exclusively hyperpolarizing photoreceptors, only half of which (S cells) produce axonal spikes in response to shadows (Wilkens, 1984). The presence of these off receptors is consistent with *Tridacna*'s predominant behavioral sensitivity to shadows.

More interesting is the fact that *Tridacna* apparently lacks photoreceptors equivalent to the depolarizing on-sensitive cells of the scallop, but yet has a direct behavioral reflex associated with a light stimulus, the orientation response. Regardless of whether this behavior represents a protective reflex or a positive phototropic response, it must originate from receptors which either fall silent when illuminated (S cells), or which synaptically influence second-order neurons in the retina. The latter alternative would provide a role for the non-spiking receptors (NS cells) which are equally represented in the retina (Wilkens, 1984). The remaining possibility is that extra-retinal photoreceptors, such as the "dermal" cells in the mantle edge of *Lima* (Mpitsos, 1973), are responsible for the direct light-sensitive responses in *Tridacna*. This is unlikely as the dermal photoreceptors in *Lima* are sensitive to dimming.

The neural circuitry of the visual system in *Tridacna* is also different from that of the scallop, the only bivalve from which there is sufficient information for comparison. In *Pecten* there are no peripheral synaptic interactions, either among the receptors in the eye (Barber *et al.*, 1967), or between optic fibers and neurones in the circumoral nerve ring (Spagnolia and Wilkens, 1983). In *Tridacna*, however, local contractions in response to dimming can be elicited in small pieces of the mantle isolated from the siphon. This requires the existence of peripherally located sensorimotor connections which also may be responsible for local siphon retractions. Synchronized movements of the siphon and shell are undoubtedly coordinated by the visceral ganglion which innervates the inhalent and exhalent regions of the siphon by separate pallial nerve trunks (Stasek, 1965), and the adductor muscle by additional motor nerves.

The scallop parietovisceral ganglion contains distinct lateral lobes (Dakin, 1910) which are devoted in large part to visual functions (Spagnolia and Wilkens, 1983). Whether equivalent functional lobes exist in *Tridacna* is not known. Nevertheless, several fairly complex visual behaviors are present in the giant clam. These include the ability to discriminate both spatial and temporal features of visual stimuli. Spatial integration is evident in several forms of behavior including the orientation response and the fact that animals habituated to a shadow at one location react strongly to changes in stimulus characteristics, *e.g.*, the unscheduled passing of a cloud during the test series, a situation which occurred on several occasions.

Sensitivity to the temporal characteristics of visual stimuli is illustrated again in animals habituated to periodic (2-min intervals) shadows. Here, vigorous reflex behaviors occur when the shadow becomes stationary. It is interesting that *Tridacna*, although extremely sensitive to moving objects, is also sensitive to a non-moving visual stimulus. Animals will react repeatedly to the interruption of an overhead light source (fiber optic light guide) that has no movement component. On the other hand, scallops respond poorly to non-moving stimuli such as a decrease in general illumination (pers. obs.).

Tridacna has evolved a unique and highly specialized visual system in association with its rather unusual hypertrophied siphon. The numerous eyes distributed along the siphon perimeter constitute a mosaic visual apparatus that is sensitive to shadows or movements in the vicinity of the animal. In addition, the visual system appears to mediate phototropic responses as an adaptation to the presence of symbiotic algae, and has the capability for discriminating spatial features of the environment. *Tridacna* is also capable of aiming the exhalent jet of seawater in the direction of a tactile stimulus (Stasek, 1965), a response observed during the present study. McMichael (1974) hints that, with the exception of *T. gigas,* tridacnids are also able to aim exhalent jets on the basis of visual information. The capacity for spatial discrimination has been established in the present study, but whether *Tridacna* actually directs its siphon on the basis of a visual pattern awaits confirmation.

ACKNOWLEDGMENTS

Space and facilities were generously provided by the Australian Institute for Marine Science in Townsville. The assistance of Martin Jones and Langdon Quetin, and the encouragement of David Sandeman, are greatly appreciated. This is a contribution (No. 244) from the Tallahassee, Sopchoppy and Gulf Coast Marine Biological Association. Supported in part by a grant from the Whitehall Foundation.

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LIFE HISTORY AND ENVIRONMENT IN TWO SPECIES OF INTERTIDAL BARNACLES

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ABSTRACT

The reproductive ecology of *Chthamalus anisopoma* and *Tetraclita stalactifera* is compared in order to understand how their life histories function in the context of their present environment, and what selective forces might have shaped their life histories. *C. anisopoma* is common in the lower intertidal of the Gulf of California. It initiates breeding six weeks after settlement; brooding and recruitment is year-round. Growth after settlement is rapid for two months, then slows markedly. Survivorship is fairly constant at all ages. Observations indicate that most mortality results from predatory gastropods, and from intraspecific crowding. *T. stalactifera* is generally confined to the upper intertidal. It failed to brood during the study period. The ovarian cycle indicates that brooding would otherwise take place during the summer. The age of first reproduction is estimated at two years. Recruitment of cyprids is strongly seasonal, peaking during August. The growth rate is relatively constant. Survivorship is initially very poor, apparently due to desiccation stress, but improves substantially after four months.

An examination of the intertidal positions and life histories of *Tetraclita* and *Chthamalus* congeners from different coastlines of the world reveals a relative conservatism of life histories, regardless of position in the intertidal. This conservatism is discussed as a general phenomena in *Chthamalus* and *Tetraclita,* and is contrasted with the relative diversity exhibited in another genus of barnacles, *Balanus.*

INTRODUCTION

In this paper I compare the reproductive ecology of *Chthamalus anisopoma* Pillsbry and *Tetraclita stalactifera* Lamarck, two species of rocky intertidal barnacles which occur at different intertidal elevations. The effect of exposure in the rocky intertidal zone is often reflected in the patterns of mortality at different intertidal levels. Abiotic factors such as desiccation stress are often a primary source of mortality at higher shore levels, while the relatively favorable conditions in the lower intertidal are associated with a predominance of biotic interactions such as competition for space, and predation (Connell, 1972, 1975; Wethey, 1983; but see Underwood and Denley, 1984, for critique). Given that organisms differ in age-specific reproductive effort, growth, and mortality, it is reasonable to assume that the differences between the low and high intertidal zones might be manifested in contrasting life history traits between ecologically similar high and low intertidal species.

The bulk of this paper is the presentation of reproductive, growth, and survivorship data gathered during a 13 month field study. The remainder of the work emphasizes how the barnacle's life histories function to allow persistence in their present envi-

Received 12 August 1985; accepted 26 February 1986.

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ronments, and asks if we can explain the selective forces that have shaped their life histories. With regard to the latter, I argue that the life histories of *C. anisopoma* and *T. stalactifera* are relatively constrained, and better understood as products of their phylogenetic histories than as adaptations to contemporary local environments (Gould and Lewontin, 1979; Stearns, 1982, 1984).

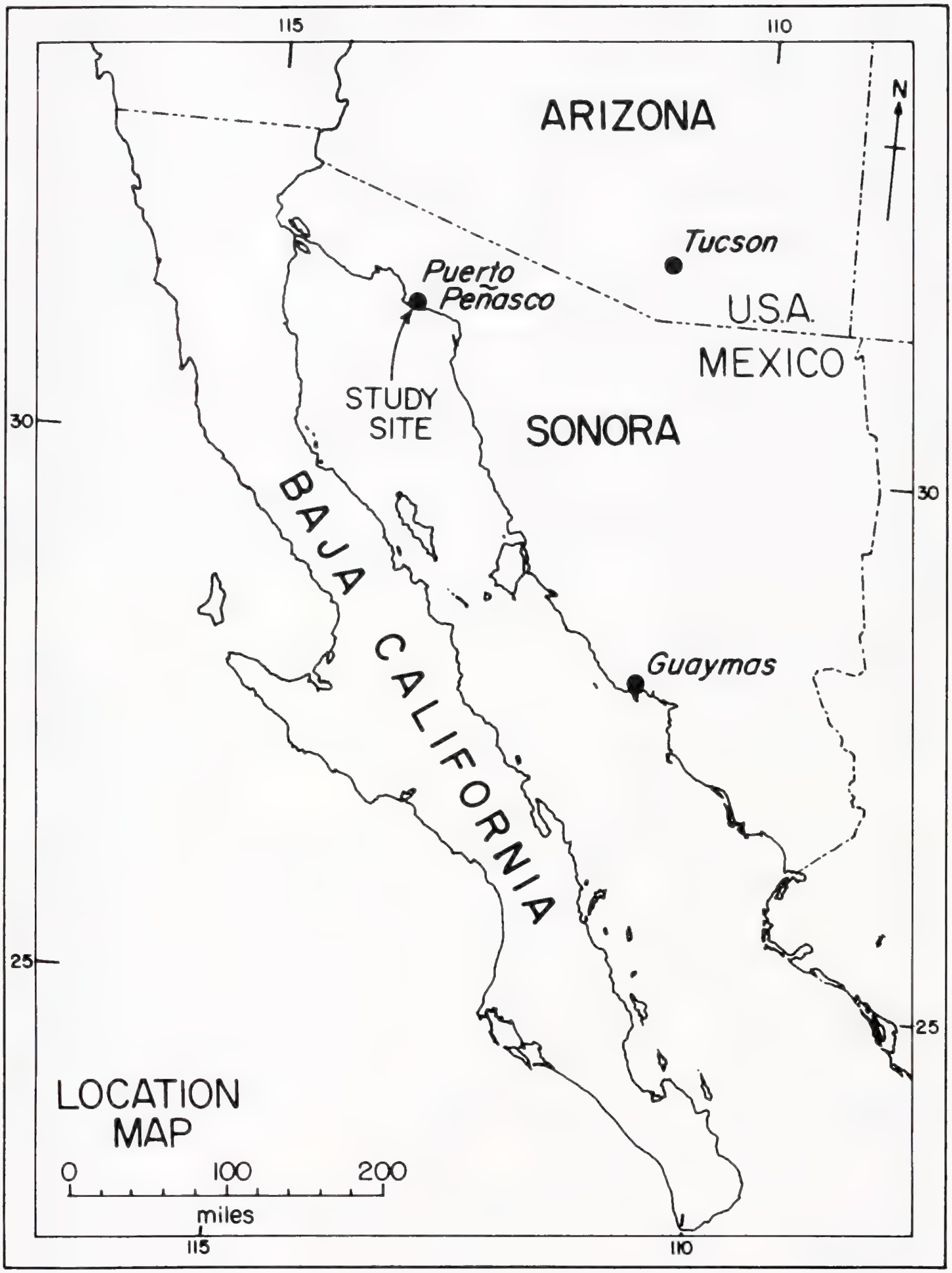
Some key life history parameters include the timing and duration of the reproductive effort, patterns of recruitment, and age-specific survivorship and growth (Stearns, 1976, 1977). These parameters are relatively easy to measure in acorn barnacles (*e.g.*, Connell, 1970, Hines, 1978). Acorn barnacles are sessile, simultaneous hermaphrodites that brood their eggs. The timing and duration of reproduction can be ascertained from sequential samples; recruitment can be quantified, and the new individuals can be mapped and followed through time to measure age-specific growth and mortality. Combining information from growth rates and dissections of small individuals, the age of first reproduction can be approximated. At present the planktonic larval stage of barnacles precludes the direct measurement of mortality before settlement and metamorphosis. The fate of larvae released from a particular local population and the geographic origin of new recruits cannot yet be determined with certainty.

MATERIALS AND METHODS

C. anisopoma is a small barnacle, with a maximum basal diameter of 6 to 7 mm. *T. stalactifera* is relatively large, reaching a basal diameter of 30 to 40 mm, and generally occurs higher on the shore (Dungan, 1985). Both species are endemic to the Gulf of California, which extends approximately 1200 km latitudinally from 23°N to about 32°N. The study site is located on open coast approximately 10 km northwest of the town of Puerto Penasco, Sonora, Mexico, (31°18″N, 113°35″W) on the mainland side of the northern Gulf of California (Fig. 1). The northern Gulf has a great tidal range (7 m spring tides at the study site), and large seasonal fluctuations in sea temperature owing to the influence of the adjacent Sonoran desert (Maluf, 1983). The study site is a grantic promontory facing west, exposed to wave action, with a broken topography composed of numerous angular boulders and sloping benches. It was visited at monthly intervals from January 1982 to February 1983. Each time samples were collected, and permanent quadrats were either photographed or censused.

Because it is not known if *C. anisopoma* and *T. stalactifera* are obligatory cross-fertilizers, specimens were collected where individuals were common and close enough together to ensure the availability of a potential mate. Samples were preserved in 70% isopropanol and brought to the laboratory, where 50 individuals of each species were selected for dissection. All size classes were represented. During each dissection I recorded the rostral-carinal axis of the opercular opening to the nearest 0.05 mm, the condition of the ovary, the presence or absence of eggs brooding in the mantle cavity, and the developmental stage of the brooding eggs. Ovaries were classified as either ripe or unripe. Ripe ovaries contained unfertilized eggs in sufficient quantity as to be visible to the unaided eye, and a considerable amount of yellow ovarian tissue bulging out into the mantle cavity; unripe ovaries lacked these qualities. Under this scheme an unripe ovary may be partially undeveloped or entirely undeveloped. Oviposition of the eggs to the mantle cavity for brooding takes place immediately after copulation (Crisp and Davies, 1955). Brooding eggs were classified as either late stage, with the nauplius eyespot visible under 10× magnification, or as early stage, lacking a visible eyespot.

To assess recruitment, growth, and survivorship, 33 permanently marked 100 cm^2 quadrats were placed at intertidal levels over nearly the entire vertical range of *C.*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N
Tucson
Puerto
Peñasco
U.S.A.
MEXICO
STUDY
SITE
SONORA
30
30
BAJA CALIFORNIA
Guaymas
25
25
LOCATION
MAP
0
100
200
miles
115
110

FIGURE 1. The study site, 10 km NW of Puerto Penasco.

anisopoma and *T. stalactifera*. These quadrats were photographed monthly, using a fixed focal length and focus to ensure the same perspective. Accuracy was checked against an included scale. The most common measure of growth in barnacles is the increase of basal diameter through time (*e.g.,* Crisp, 1960). This was not a suitable measurement for either *C. anisopoma* or *T. stalactifera* because in both species the base was often obscured by neighboring individuals. Instead, the growth of individual barnacles was followed through time using a dissecting scope fitted with an ocular micrometer to measure, to the nearest 0.1 mm, the rostral-carinal axis of the opercular opening on the photograph. The relationship between wet body weight and the opercular opening was determined via the measurement and dissection of 30 animals of each species.

Recruitment of juveniles was quantified by censusing in the field three groups of five 25 cm^2 plots at three intertidal levels: the *C. anisopoma* zone (+0.6 m above Mean Low Water), the lower *T. stalactifera* zone (+1.5 m), and the upper *T. stalactifera* zone (+2.3 m). After censusing, the plots were cleared with a wire brush and a sodium hydroxide solution (Easy-Off oven cleaner) to provide a clean substrate for cyprids. The oven cleaner was washed away with the incoming tide and did not appear to affect settlement. It is important to note that the plots were cleared each month. If the plots were left uncleared the number of cyprids settling might become a function of available space rather than an indication of what is available for settlement (Connell, 1961b; Meadows, 1969). Still, this measure is only a minimal estimate of settlement during the preceding month. While there is often a significant, positive correlation between settlement density and number of survivors (recruits), at high settlement densities the proportional survival drops, suggesting density-dependency at high densities, but not low (Caffney, 1985; Connell, 1985).

Age-specific survivorship was assessed from the photographed quadrats by placing a sheet of clear acetate over the photograph and placing a dot over each individual barnacle to be followed. The clear sheet could then be placed over the next month's photograph, and so forth, to check for the presence or absence of each marked barnacle. Deaths were noted by either the complete absence of the barnacle or by missing opercular valves.

Survivorship of *C. anisopoma* was followed after a large recruitment in April and May of 1982. Four hundred and twenty juveniles were chosen from the photographs on the basis of image clarity and followed for the next nine months. Growth data was used to infer an approximately 2.5–2.8 mm (opercular diameter) size for barnacles aged nine months. Barnacles of this size class (here termed the 1981 year class) were followed from age 9 months to 20 months to extend the survivorship information. For *T. stalactifera,* survivorship after recruitment was followed after the first and largest set in August of 1982. Five hundred and fifty-eight individuals that settled during this period were mapped and followed on photographs for the next six months. A size frequency histogram of all *T. stalactifera* in photographed quadrats allowed identification of the 1981 and 1980 year classes. The survivorship of these year classes was followed to extend the survivorship information.

RESULTS

Reproduction

Ovarian cycles and brooding frequencies of *C. anisopoma* are shown in Figure 2. Ripe ovaries were found throughout the year. With the exception of a peak of 90% in July, there was no clear seasonal pattern of ovarian development. Brooding also occurred year-round, but with a significant drop during the summer. In June and July, 44% to 52% of the sample contained developing embryos; in January, February, and December of 1982, and January and February of 1983, the percentage brooding fluctuated between 76% and 94%.

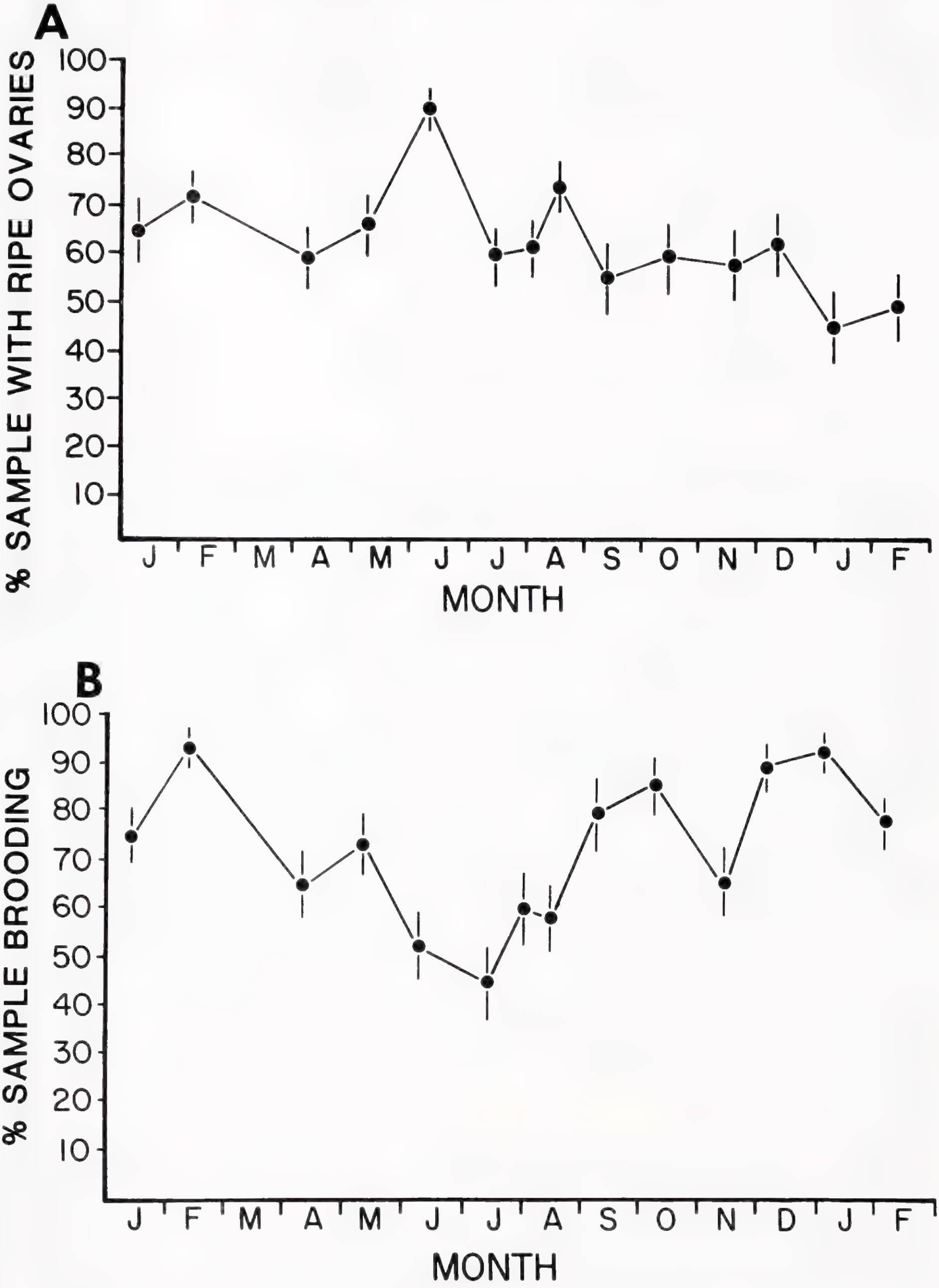


FIGURE 2. (A) The ovarian cycles and the (B) brooding frequencies in *Chthamalus anisopoma*. Bars indicate standard error (n = 50/month).

During each dissection of *C. anisopoma* the condition of the ovary relative to the developmental stage of the brooding embryos was recorded; this information is compiled in Table I, which sums the information for all samples of *C. anisopoma* over

TABLE I

[illegible]*al stage of ovary relative to brooding stage in* Chthamalus anisopoma; *e.g., 91%* [illegible]*ted animals not brooding had ripe ovaries*

	Ovary condition	
Stage of brooding	Not ripe (%)	Ripe (%)
Not brooding n = 193	9	91
Early stage n = 370	61	39
Late stage n = 137	11	89

the entire study. Note that 89% of those samples found with "late stage" embryos, *i.e.*, eyed nauplii, had ripe ovaries. This suggests that the ovaries redevelop rapidly while the brood is developing in the mantle cavity, and that the brood's rate of development may be controlling the length of time between broods, at least when food is available.

The smallest *C. anisopoma* sampled that contained brooding eggs had an opercular opening of 1.25 mm. Individuals in the size class of 1.45 to 1.60 mm were brooding at a significantly lower frequency than the remainder of the population ($\chi^2 = 10.06$, $.005 > P > .001$); individuals of the size class 1.65 to 1.80 mm were brooding at frequencies not significantly different from the remainder of the population ($\chi^2 = 3.81$, $.10 > P > .05$). Hence, individuals with an opercular opening greater than 1.65 mm were judged to be reproductively mature. This corresponds to an age of about six weeks (see growth data, below).

The ovarian cycle of *T. stalactifera* is distinctly seasonal and synchronous within the sampled population (Fig. 3). The percentage of individuals with ripe ovaries climbs steeply during the spring, reaches its peak in early August, and then drops precipitously. Almost no individuals could be found with ripe ovaries during the winter.

Only three *T. stalactifera* individuals in the entire sample of 740 individuals were brooding eggs, one each in June, July, and August. The opercular diameters of the three individuals were 3.70, 4.80, and 5.65 mm. An individual with an opening of 3.70 mm is approximately 20 months of age (see below).

Recruitment

While *C. anisopoma* readily settled on cleared plots, *T. stalactifera* did not, instead preferring to settle on the tests of conspecifics. Consequently, only the photographed plots could be used to assess *T. stalactifera* recruitment, and the cleared plots were used for *C. anisopoma* recruitment. The data are derived from different sources, and quantitative comparisons between *C. anisopoma* and *T. stalactifera* are inappropriate.

The average number of *C. anisopoma* recruits each month on cleared plots is shown in Figure 4a. Because the plots were not cleared until April, recruitment for February through April was estimated by examination of photographed quadrats. There was light recruitment during this period, averaging about 50 individuals per 25 cm^2. Recruitment appeared to increase gradually from February to April.

Recruitment was very heavy from April to September on cleared plots in the *C. anisopoma* zone, with a maximum of nearly 600 individuals per 25 cm^2 during

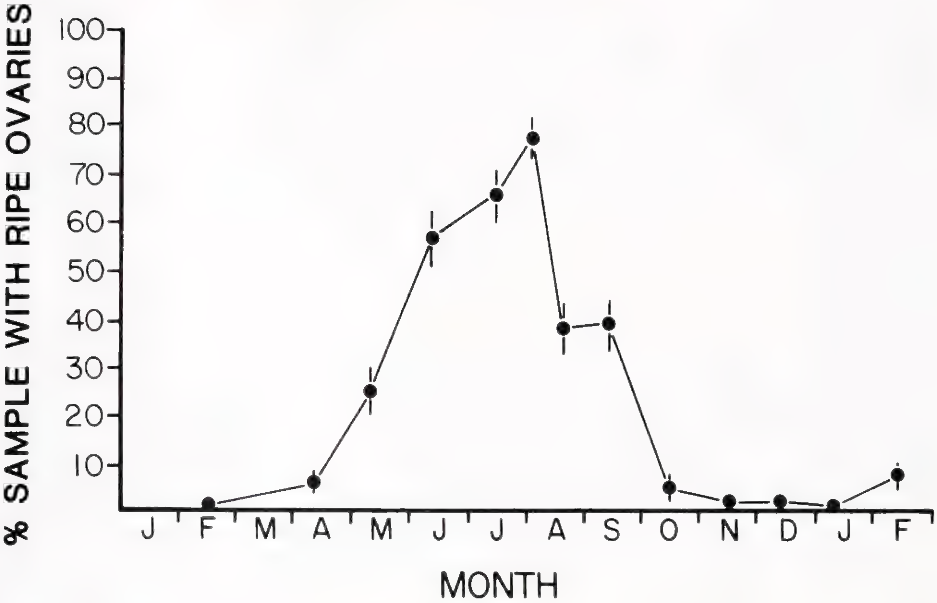


FIGURE 3. The ovarian cycles in *Tetraclita stalactifera.* Bars indicate standard error (n = 50/month).

the late summer. Recruitment then decreased until in November and December no juveniles were noted in the cleared plots, even though there was light recruitment noted in some of photographed quadrats. Recruitment then increased again in January and February of 1983. The cleared plots in the lower *T. stalactifera* zone received substantial *C. anisopoma* recruitment only during the month of August, which coincided with the peak of recruitment in the *C. anisopoma* zone. *C. anisopoma* never recruited in the upper *T. stalactifera* plots.

Average *T. stalactifera* recruitment in photographed quadrats is shown in Figure 4b. There was a sharp seasonal peak in August, followed by a steady decline in September and August. Only a few scattered juveniles could be found in November. No recruitment occurred on the ten cleared plots in lower and upper *T. stalactifera* zone, even during the period of heaviest recruitment. I noted that when *T. stalactifera* did settle directly on the rock substrate, it preferentially settled on scars left by the recently detached tests of conspecifics.

Growth

The growth of thirty-four newly settled *C. anisopoma* individuals from three adjacent quadrats at two different tidal levels (both in the *C. anisopoma* zone) was followed from April 1982 to February 1983. Individuals were chosen on the basis of clarity of image in photographs. Data from individuals in the higher quadrats are shown in Figure 5a. After eight to nine months many *C. anisopoma* had died, resulting in the smaller sample sizes and larger confidence intervals. A summary of growth data for *C. anisopoma* is shown in Figure 5b. Visual inspection of growth curves suggests no consistent differences between individuals at the two different tidal levels. All individuals grew quickly for the first two months before sharply leveling off.

Data for the growth of newly settled *T. stalactifera* was obtained in a manner

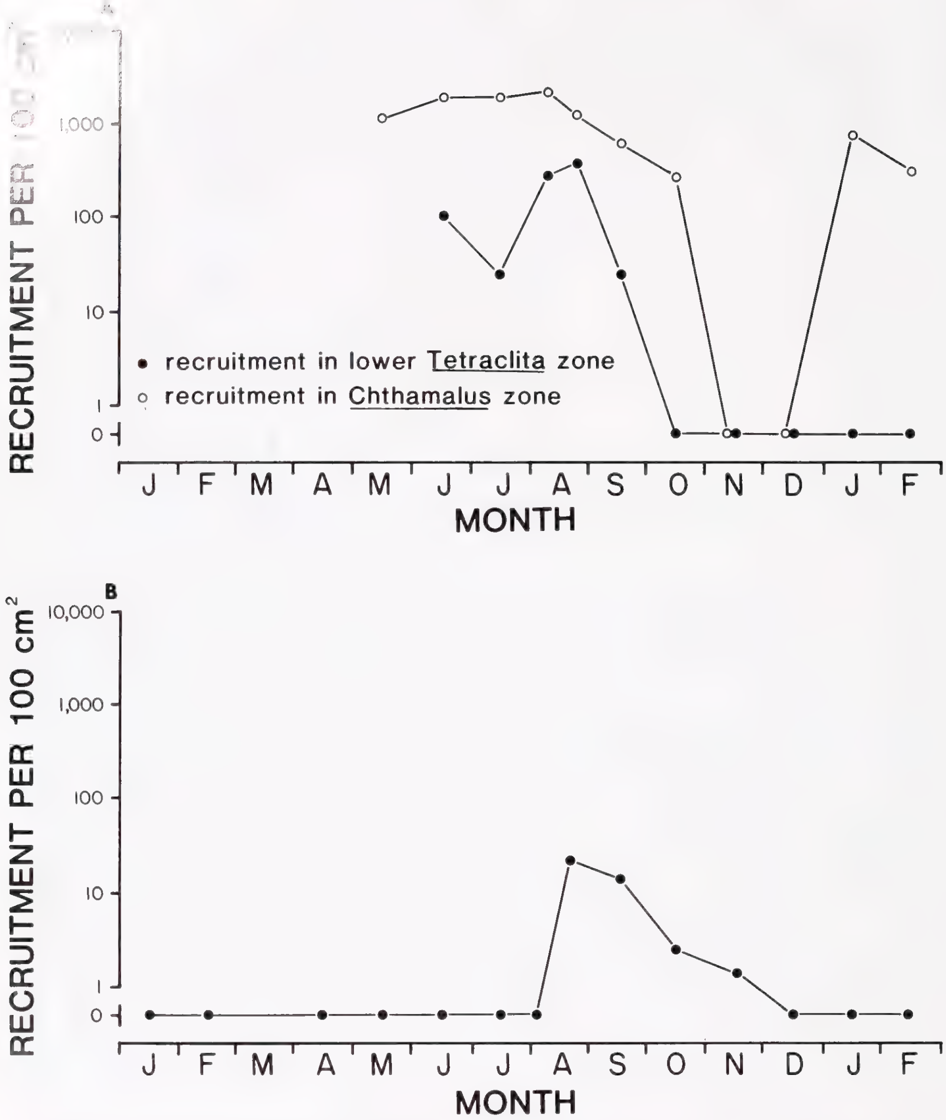


FIGURE 4. (A) The recruitment of *Chthamalus anisopoma* in cleared 25 cm^2 plots and the (B) recruitment of *Tetraclita stalactifera* in photographed 100 cm^2 quadrats.

similar to that described for *C. anisopoma*. An example of growth rates is shown in Figure 6a. Growth rate information was extended by measuring the growth of the 1981 year class individuals, as identified from a size frequency histogram of all measurable *T. stalactifera* in photographed quadrats. An example of the growth rates of 1981 year class individuals is shown in Figure 6b. Data from both newly settled and 1981 year class individuals are compiled in Figure 6c, which summarizes approximately 19 months of growth. All data were from areas of similar exposure and elevation. For further growth data see Malusa (1983).

Survivorship

The survivorship data for each species was summed and adjusted to 1000 newly settled individuals to allow comparison between *C. anisopoma* and *T. stalactifera* survivorship. The survivorship of the 1981 and 1980 (*T. stalactifera* only) year classes are treated as extensions of the initial curve derived from the newly settled individuals. Figure 7 shows the adjusted survivorship of *C. anisopoma* and *T. stalactifera* plotted from an arbitrary 1000 individuals.

DISCUSSION

Reproductive ecology

Chthamalus anisopoma initiated breeding six weeks after settlement, and brooded continuously thereafter, year-round and in sea surface temperatures ranging from approximately 13°C to 33°C. *C. fissus, C. stellatus,* and *C. dentatus* (Klepal and Barnes, 1975; Hines, 1978) also produce a large number of broods, but these are usually limited to the warmer months of the year. For example, Hines (1978) estimates that *C. fissus* along the western United States coast produces 16 broods from March or April to October. Laboratory experiments show that development of the ovary is generally limited by food availability, while the rate of brood development is a function of temperature (Crisp, 1950; Patel and Crisp, 1960a, b). When food is available, reproduction is virtually continuous, with the hatching and release of one brood followed by the deposition of another (Hines, 1976). That 91% of those *C. anisopoma* not brooding had ripe ovaries apparently ready for deposition (Table I) provides strong circumstantial evidence for similar continuous reproduction.

C. anisopoma recruited throughout most of the year, with a maximum in August and a minimum in November and December. *C. fissus* also recruits throughout the year, and at all intertidal levels (Hines, 1979). *C. anisopoma* recruitment was largely limited to the *Chthamalus* zone, recruiting higher only during August, when most space in the *Chthamlus* zone had already been pre-empted by other *C. anisopoma.*

C. anisopoma grew quickly for the first two months, after which growth dropped off dramatically. The sharp decline in the growth rate occurs shortly after the estimated age of first reproduction of six weeks. Crowding may also play a role in the change in growth rate, but there were no controls for comparison to substantiate this. *Elminius modestus* in the northeastern Atlantic has a similar pattern of growth. It begins reproducing at about two months of age, after which growth is sharply curtailed (Crisp, 1960). The early age of reproduction is indicative of the relatively short life span of *C. anisopoma.* The largest individual in photographed quadrats had an opercular diameter of 3.85 mm. Extrapolating from the growth rate in Figure 6, this corresponds to an age of approximately three years.

The mortality rate of *C. anisopoma* in photographed quadrats was fairly constant at all ages. Eighty percent of the newly recruited individuals survived at least six weeks, when reproduction begins. Adults were frequently consumed by the gastropods *Acanthina angelica* and *Morula ferruginosa,* barnacle predators common in the lower intertidal (Paine, 1966; Dungan, 1984; Malusa, 1985). Also, sequences of photographs revealed that *C. anisopoma* was often a victim of its own growth. Intraspecific competition for space resulted in the formation of hummocks, raised mats of barnacles, which eventually were dislodged by wave action. Connell (1961b) documents a similar source of mortality for *Semibalanus* (= *Balanus*) *balanoides* off the coast of Scotland.

The ovaries of *T. stalactifera* showed distinct seasonal variation, developing as the

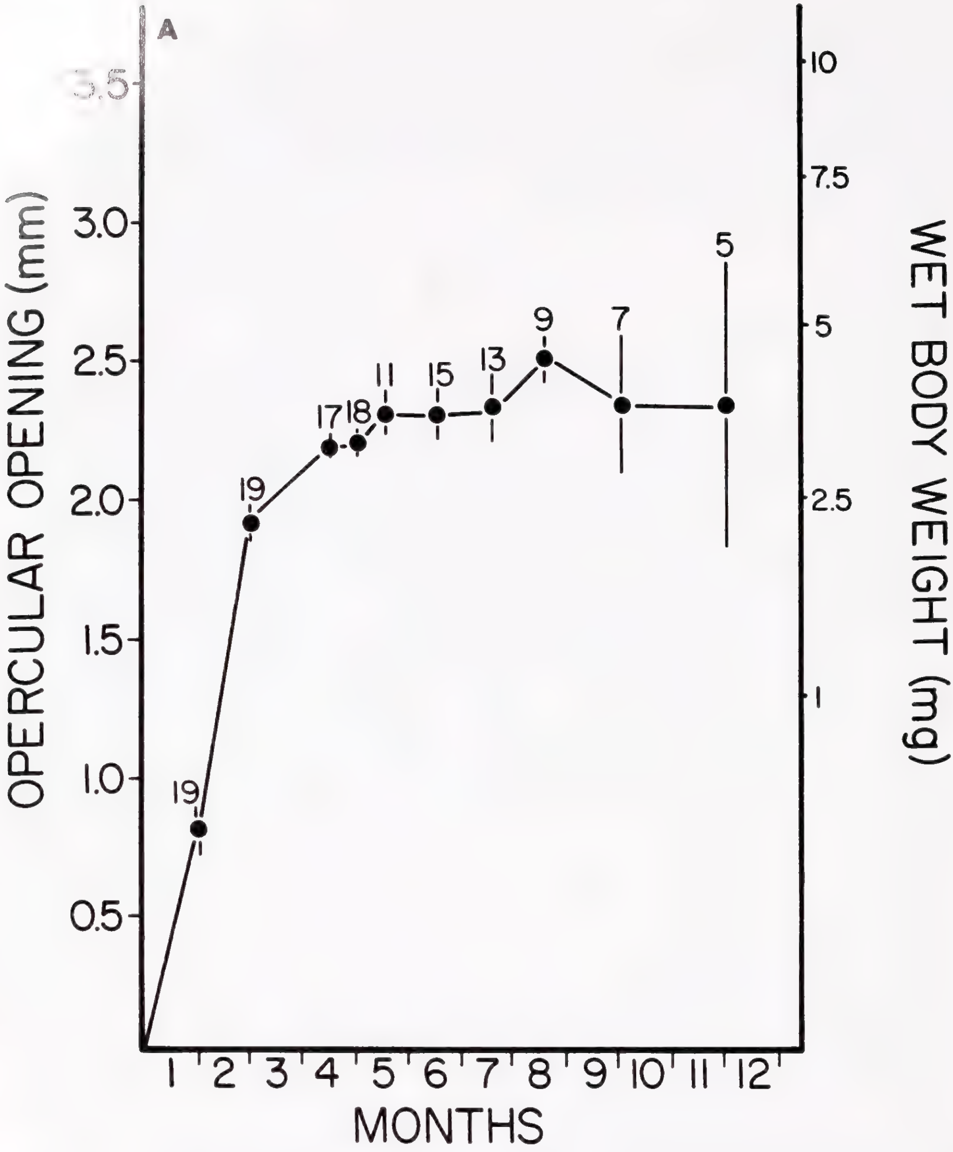


FIGURE 5. (A) The growth from settlement of *Chthamalus anisopoma* from three photographed quadrats in the upper *Chthamalus* zone. Means, sample sizes, and 95% confidence intervals are indicated. (B) Growth summary for *Chthamalus anisopoma,* plotting the means at all ages from two groups of three quadrats each. Wet body weight is inferred from the regression equation: Weight (mg) = 0.49 (length [mm])$^{2.34}$. $r^2 = 0.88$.

sea temperatures rise in the spring and summer. Hines (1978) found a similar pattern in *Tetraclita rubescens* (= *T. squamosa*) along the western U. S. coast. Although *T. stalactifera* apparently failed to brood during this summer, the ovarian cycles indicate that brooding would otherwise take place during the summer, as it did in *T. rubescens.*

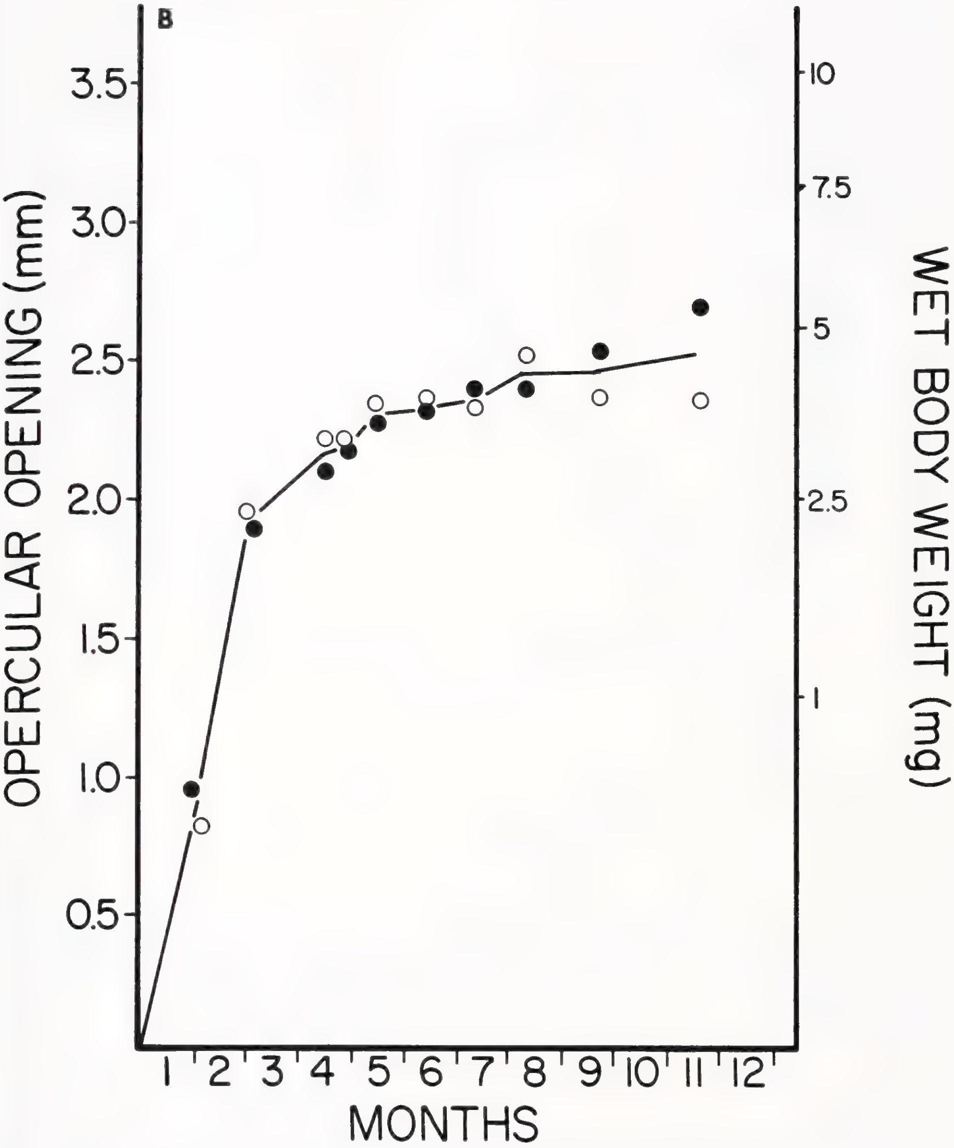


FIGURE 5. (*Continued*)

Only 3 *T. stalactifera* out of 740 dissected contained brooding eggs. Related incidents observed in other species of *Tetraclita* suggest that reproduction in *Tetraclita* is highly variable and supports the hypothesis that reproduction failed to occur in the population under study. Hines (1978) attempted to elicit brooding in laboratory of *T. squamosa* (= *rubescens*). Though similar lab populations of *Chthamalus fissus* and *Balanus glandula* brooded, the *Tetraclita* did not, developing only ripe ovaries. Villalobos (1980) sampled monthly a population of *T. panamensis* in Costa Rica and found that the first year individuals all had "mature ovaries," but did not reproduce

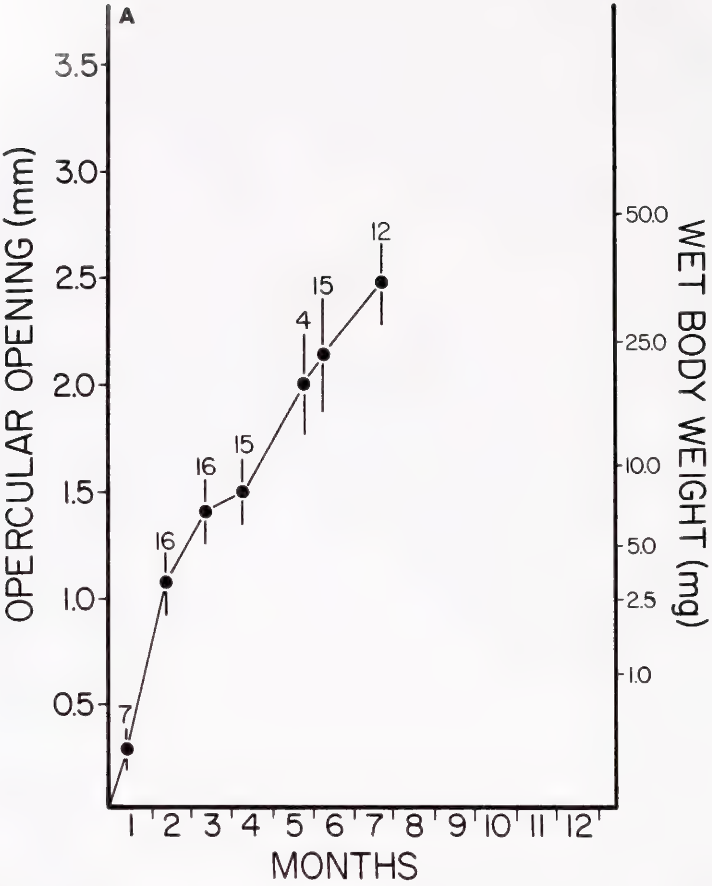
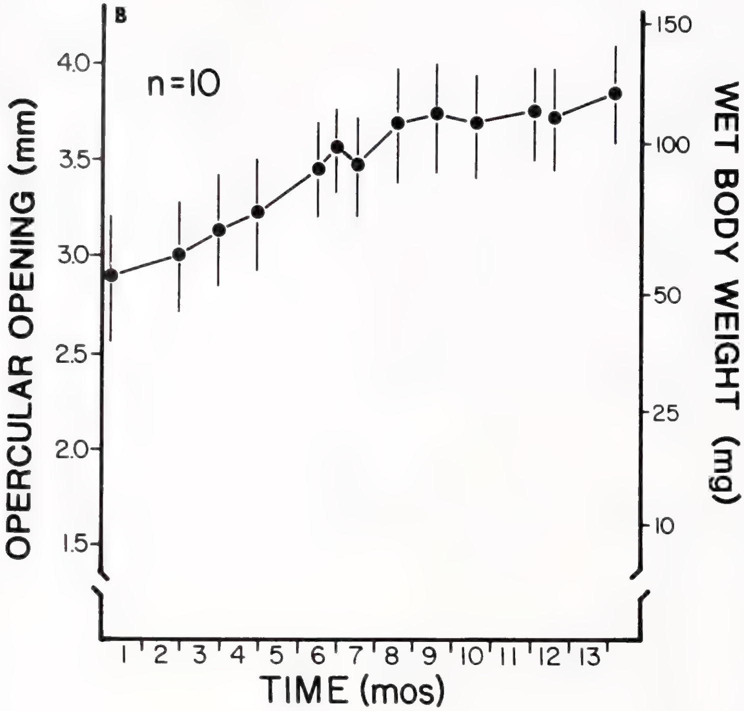


FIGURE 6. (A) The growth from settlement of *Tetraclita stalactifera* from three photographed quadrats in the lower *Tetraclita* zone. (B) The growth of the 1981 year class of *Tetraclita stalactifera* in two quadrats from the lower *Tetraclita* zone. Means, sample size and 95% confidence intervals are indicated. (C) Growth summary for *Tetraclita stalactifera*, plotting the means for all ages from five samples (*e.g.*, Fig. 6A represents a single sample). Wet body weight is inferred from the regression equation: Weight (mg) = 2.7 (length [mm])$^{2.8}$. $r^2 = 0.92$.

until their second year. Even though Villalobos' grading of ovary ripeness is difficult to interpret, it is clear that there was development of the ovary without subsequent brooding. Achituv and Barnes (1978) maintained laboratory populations of *T. stalactifera rufotincta*. In July the samples had ripe ovaries with visible unfertilized eggs. In August, the condition of the ovaries regressed, yet none of the animals were brooding embryos. This is the closest parallel to the data in this study.

Recruitment of *T. stalactifera* cyprids was predominantly on the tests of conspecifics, a gregarious behavior also noted in *Tetraclita squamosa* of the Caribbean Sea (Birnbaum and Zischke, 1979). Settlement was heaviest in August and September, and had ended by November. A minority of individuals settled on bare substrate at all intertidal levels, albeit in reduced numbers.

The recruitment of barnacle cyprids in a given population may be interpreted as an indication that brooding has taken place in the same or nearby population. However,



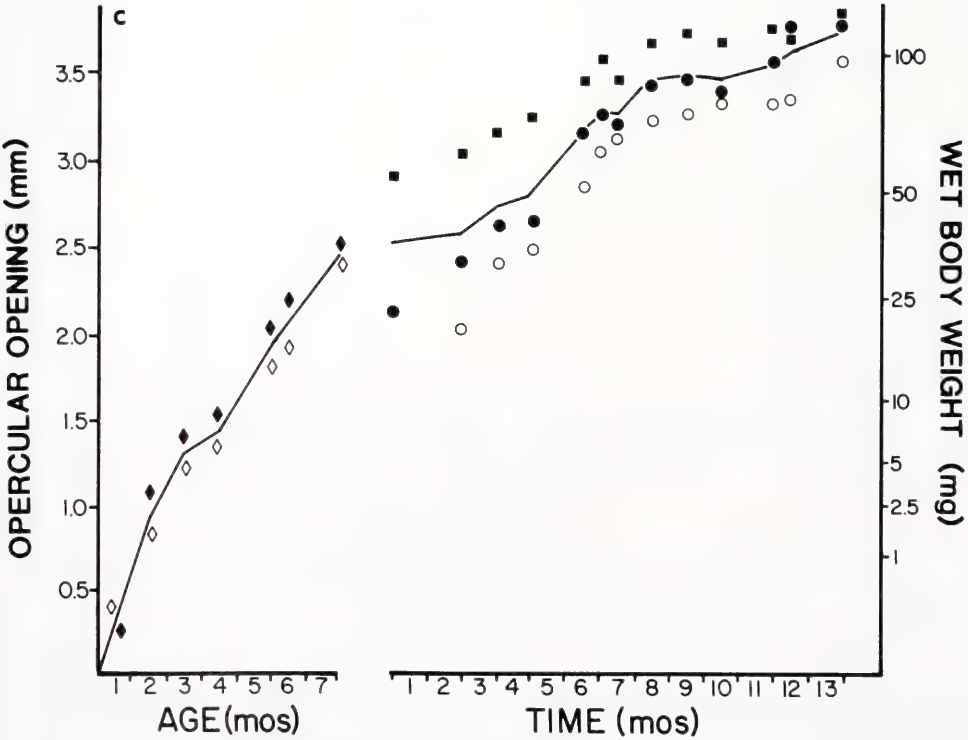


FIGURE 6. (*Continued*)

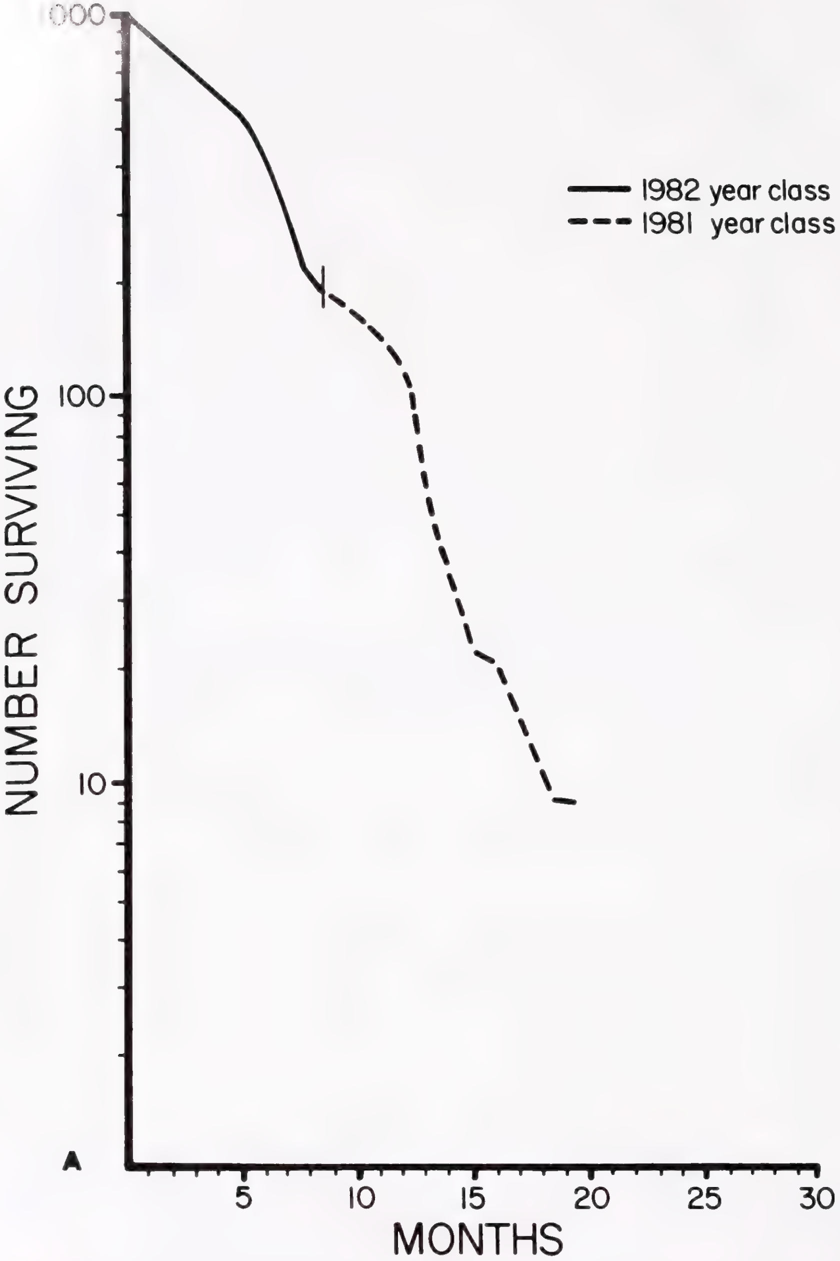


FIGURE 7. (A) Adjusted survivorship of *Chthamalus anisopoma* recruited during April and May from all photographed quadrats. (B) Adjusted survivorship of *Tetraclita stalactifera* recruited during August from all photographed quadrats.

planktonic larvae may travel great distances; their origin is usually unknown. Wethey (1980) estimated that the sibling larvae of a single *Balanus balanoides* individual may settle along 50 km of coast in a period of several days. In my study population there

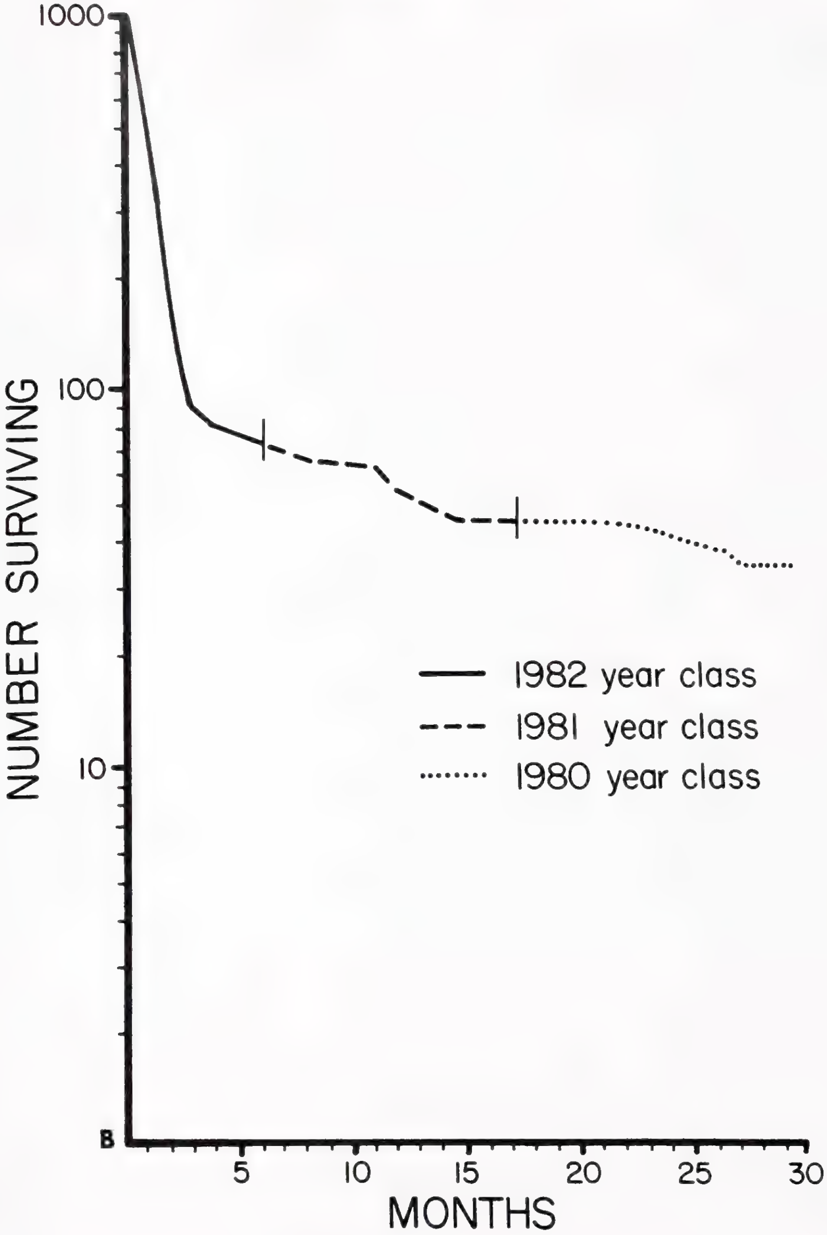


FIGURE 7. (*Continued*)

was substantial recruitment even though there was little reproduction. Other studies on *Tetraclita* spp. show a poor correspondance between brooding in a study population and recruitment in the same. *Tetraclita rubescens* recruited approximately three months after brooding (Hines, 1979), *T. stalactifera rufotincta* of the Red Sea recruited during the last month of brooding (Achituv and Barnes, 1978), and *T. panamensis* of the tropical eastern Pacific recruited during the same month that brooding activity initiated (Villalobos, 1980).

The growth rate of *T. stalactifera* was relatively constant; the distinct change in growth rate observed for *C. anisopoma* was lacking. Assuming a growth rate similar to that of second-year individuals, the largest individual in photographed quadrats is estimated to be 8 to 9 years old. The age of first reproduction was estimated to be about two years, based on the single smallest individual found brooding. *Tetraclita panamensis, T. rubescens,* and *T. stalactifera rufotincta* all have an estimated age of first reproduction of about two years (Hines, 1976, 1979; Achituv and Barnes, 1978; Villalobos, 1979, 1980).

T. stalactifera suffered relatively high initial mortality, with only 8% of the newly recruited animals surviving to four months of age. But survivorship improved considerably with age: 43% of the 4-month-olds will live to at least 28 months of age. The high initial mortality rate is likely a consequence of a high intertidal habitat and its associated desiccation stress. Peak settlement is during August, the hottest month of the year. Adult *T. stalactifera* are highly desiccation resistant (Dungan, 1985) and the causes of deaths in older individuals are not clear. Some were observed to be drilled by the gastropod *Acanthina angelica,* even though this snail's range is primarily centered over the *Chthamalus* zone. The high intertidal habitat of *T. stalactifera* may be a more effective refuge from predation than its size, as no gastropod or asteroid predators were ever observed near the top of its range. Connell (1970) found a similar spatial refuge from predatory gastropods with the barnacle *Balanus glandula* in the northeastern Pacific.

In general, barnacle species that inhabit the upper intertidal are more desiccation resistant than those typical of the lower intertidal (Barnes and Barnes, 1957; Foster, 1971, 1974). In an experiment where samples of *C. anisopoma* and *T. stalactifera* were transplanted above the high tide line, Dungan (1985) found that 54 out of 100 *T. stalactifera* adults survived 3 days of complete exposure in temperatures up to 35°C; only 3 out of 100 *C. anisopoma* adults survived half as long. Physiologically, *T. stalactifera* is able to survive in the lower intertidal, as evidenced by the presence of scattered individuals in the *Chthamalus* zone. But *C. anisopoma* apparently excludes *T. stalactifera* in the lower intertidal by the pre-emption of free space, and through the direct effects of crowding. Dungan (1985) showed *C. anisopoma* to reduce the growth and survival of juvenile *T. stalactifera* that settled in the *Chthamalus* zone, relative to those experimentally freed of the effects of *C. anisopoma.* Also, *C. anisopoma* effectively monopolizes space by virtue of its early and constant reproduction and heavy settlement. This is especially so in the late summer. When *T. stalactifera* began settlement in August, *C. anisopoma* had already covered virtually all the substrate in the lower intertidal (94%, SD = 3%), leaving little space for further settlement (I have never observed either species to settle on *C. anisopoma* tests, but both will settle on *T. stalactifera* tests). A similar *C. anisopoma–T. stalactifera* interaction is lacking in the upper intertidal zone, where *C. anisopoma* is evidently excluded by desiccation stress.

Life histories as adaptations: cause and effect

Assuming that natural selection acts upon variation in age-specific reproductive effort, growth, and mortality, life history theory attempts to predict what suite of traits should be selected for in a given environment (Stearns, 1976, 1977). Within this framework, a species' schedule of reproduction and growth is often interpreted as an adaptation to the biotic and abiotic components of the environment (*e.g.,* Hines, 1979).

The intertidal can be characterized as a gradient of increasing exposure from a relatively stable environment in which competition and predation are an important source of mortality, to a physically rigorous and physiologically limiting environment. A comparison of mortality in *C. anisopoma* and *T. stalactifera* supports this generalization. In the lower intertidal, the relatively high levels of recruitment in *C. anisopoma* led to considerable intraspecific crowding, which often resulted in hummocking and subsequent death. Predation intensity is generally greater at lower shore levels (*e.g.*, Connell, 1961a). At higher shore levels, the very high initial mortality of *T. stalactifera* is probably density-independent, resulting from desiccation stress. The case for density-independence is supported by the very low recruitment densities (see Fig. 7b), and the relative lack of predators such as the gastropod *Morula ferruginosa* in the upper intertidal at Puerto Penasco.

Accepting the above characterization of the intertidal for comparative purposes allows examination of hypotheses regarding the evolutionary origin of the barnacles' life histories. For example, r- and K-selection theory (MacArthur and Wilson, 1967; Boyce, 1984 for review) predicts that a relatively stable environment with predominantly density-dependent mortality should select for increased efficiency and a higher "K", or carrying capacity. Pianka (1970) later associated a higher "K" with a later age of first reproduction and increased size and longevity. A rigorous, unstable environment characterized by density-independent mortality should select for an increase in the intrinsic rate of population growth (the logistic "r"), which can be attained most quickly by an earlier age of first reproduction (Lewontin, 1965), with an attendant decrease in longevity (Bonner, 1965). Hence, the theory would predict "r-selected" traits in *T. stalactifera,* and "K-selected" traits in *C. anisopoma.*

The results of this study show that this is not the case. *C. anisopoma* is characterized by "r-selected" traits, and *T. stalactifera* by "K-selected" traits. This disparity between prediction and data stems from the implicit assumption that increased size and delayed reproduction are superior competitive traits for limited resources. This assumption is not supported for *C. anisopoma* and *T. stalactifera* (Dungan, 1985). Space is generally the limiting resource for sessile organisms in the lower rocky intertidal (Dayton, 1971), and at Puerto Penasco, *C. anisopoma* effectively excludes *T. stalactifera* in the lower intertidal (Dungan, 1985).

An alternative theory of life history evolution, called "bet-hedging" by Stearns (1976), predicts that long life and a reduced reproductive effort will be selected for in the face of unpredictable survival of prereproductives (Murphy, 1968; Charnov and Schaffer, 1973; Schaffer, 1974). Bet-hedging may be a good explanation in this case, but requires a study of variation in reproductive success through time, which could not be determined directly in a study of this length.

Additional study should be put in a broader context, taking into account the life histories and environments of close relatives. It is significant that the relative positions of *Chthamalus* and *Tetraclita* congeners in the intertidal zone are reversed from one geographic region to the next. *Tetraclita* is above *Chthamalus* in the Gulf of California (Dungan, 1985), on the Pacific coast of Panama at Punta Paitilla (Reimer, 1976), and the Gulf of Elat, Red Sea (Achituv, 1972). *Chthamalus* is above *Tetraclita* along the California coast (Hines, 1979), and along the Florida Keys (Bierbaum and Zischke, 1979). Yet while their intertidal positions are highly variable, some of the life histories characteristic of *Chthamalus* and *Tetraclita* appear essentially conservative from one region to the next. Body size varies little within the two genera, with all the members of *Chthamalus* being smaller than all the members of *Tetraclita* (Newman and Ross, 1976; Stanely and Newman, 1980; Paine, 1981). The age of first reproduction is approximately two years for the four species of *Tetraclita* on which data are available (Hines, 1976; Achituv and Barnes, 1978; Villalobos, 1979, 1980).

The age of first reproduction in *Chthamalus* is more variable. Based on settlement records, *C. stellatus* in Europe and *C. fragilis* of the U. S. Atlantic coast first reproduce at about nine months of age (Connell, 1961a; Wethey, 1983). But the appropriate comparison for this study is between *C. anisopoma* and its closest relative, which biochemical evidence suggests is *C. fissus* of the eastern Pacific (Hedgecock, 1979). The upper intertidal species, *C. fissus,* reproduces first at approximately two months after settlement (Hines 1976); the lower intertidal species, *C. anisopoma,* reproduces first at about six weeks. This and other life history similarities (such as size and life span; see Hines, 1976) between the two species make it difficult to provide a causal explanation for the life history traits of *C. anisopoma* in terms of environmental selection forces along an intertidal gradient.

These examples of apparent conservatism in *Tetraclita* and some members of *Chthamalus* suggest that historic explanations should be considered when drawing inferences from comparative studies (Gould and Lewontin 1979; Felsenstein 1985). On the other hand, such conservatism cannot be considered a general feature of all acorn barnacles. For example, the age of first reproduction in different species of the genus *Balanus* varies from four to eight weeks (Walker, 1961; Werner, 1967; Hurley, 1973), to one to two years (Crisp, 1954; Barnes and Barnes, 1954). If *Balanus* is a monophyletic taxon, it may be a profitable group to focus on if we wish to understand the adaptive significance of a trait such as age of first reproduction. The observation that some genera appear relatively variable, and others conservative, suggests that further hypotheses of life histories as adaptations be firmly grounded in a phylogentic context.

ACKNOWLEDGMENTS

This work formed part of a thesis submitted to San Diego State University. I am much indebted to Thomas Ebert for sharing his expertise in life histories, and to Michael Donoghue for opening my eyes and mind to the importance of an organism's history. Michael Dungan was the original impetus for this project; his enthusiasm and scientific insight were a driving force. I thank Sherman Suter, Donald Thomson, and an anonymous reviewer for their criticisms of earlier drafts of this paper, and Andy Wyss, Susan Fansler, Lissa Findley, Michael Russell, and Fernando Jara for their assistance in transportation to and work at the field site. This work was supported in part by a Sigma Xi Grant-In-Aid of Research.

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TIDAL PERIODICITY IN THE DAILY SETTLEMENT OF INTERTIDAL BARNACLE LARVAE AND AN HYPOTHESIZED MECHANISM FOR THE CROSS-SHELF TRANSPORT OF CYPRIDS

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ABSTRACT

At an intertidal study site in southern California the daily settlement of barnacle cyprids (probably *Chthamalus* spp.) was followed during the summer of 1983. Daily settlement was not significantly cross correlated with wind speed or direction but was significantly cross correlated with the maximum daily tidal range at lags of +1 to +4 days; peak settling occurred several days before the spring tide. This pattern of settlement is nearly identical to that of the megalopa of an intertidal crab, *Pachygrapsus crassipes*, and this suggests that, like these megalopae, cyprids may be transported onshore in slicks over tidally forced internal waves.

INTRODUCTION

Barnacle larvae reside in the plankton for about 3 to 6 weeks (Pyefinch, 1948; Strathmann *et al.*, 1981; Harns, 1984). The terminal larval stage, the cyprid, must migrate* from its location in the pelagic environment back to the shore if it is to continue its development. The quantity of larvae settling at a location can determine the size of the adult population (Connell, 1985; Gaines and Roughgarden, 1985). Thus, the relative success of the cyprids' migration back to the coast may help determine adult abundance.

There seems to be three mechanisms for the cross-shelf movement of cyprids: (1) cyprids swim ashore, (2) chance events deposit cyprids at the shore, and (3) cyprids, by their position in the water column, exploit onshore currents which carry them back to shore.

The swimming speed (several cm/s, Crisp, 1955) and simple sensory system of cyprids would seem to exclude the possibility that they swim ashore. Studies of the daily settlement of cyprids (Bennell, 1981; Kendall *et al.*, 1982; Hawkins and Hartnoll, 1982; Wethey, 1985) suggest that cross-shelf transport is not controlled by chance events. The daily abundance of settlers may be related to the direction of wind driven currents (Bennell, 1981; Hawkins and Hartnoll, 1982) or in contrast, may vary on a lunar cycle (Wethey, 1985). The fact that settlement does not appear to be random with respect to time, but may be related to water movement suggests that cyprids most likely migrate onshore by exploiting onshore currents.

In southern California onshore flow is generated by either the winds or the tides (Winant and Olson, 1976; Winant and Bratkovich, 1981). This is probably true for

Received 25 November 1985; accepted 14 February 1986.

* The definition of migration is that employed by entomologists. "Migration is a persistent, straightened-out movement with some internal inhibition of the responses that will eventually arrest it. It may be effected by the insect's own locomotory exertions or by its active embarkment on some transporting vehicle" (Kennedy, 1961).

most coasts. Evidence that wind generated currents carry cyprids ashore is ambiguous. In one study maximum settling rates occurred during periods of onshore winds (Hawkins and Hartnoll, 1982), but in another it occurred when winds were offshore (Bennell, 1981). None of the studies of daily barnacle settling have attempted to relate the abundance of settlers to tidal phase, although Wethey's (1985) observation of a lunar cycle to settling rate (1985) could alternately have been interpreted as a tidal cycle. The possibility that the tides play a role in the onshore transport of cyprids has not been tested.

In recent papers (Shanks, 1983, 1985a, b; Kingsford and Choat, in press), evidence was presented which suggests that crab megalopae and larvae of other coastal invertebrates and fish are transported from the offshore plankton to the coastline in surface slicks generated over tidally forced internal waves. As the ebbing tide flows across bottom relief such as reefs, banks, and the continental shelf break, lee waves are generated on the thermocline (Rattray *et al.,* 1969; Halpern, 1971; Maxworthy, 1979; Chereskin, 1983). Along the continental shelf the lee wave is formed at the seaward edge of the shelf break (Fu and Holt, 1982). When the ebbing current goes slack, the lee wave is released and progresses shoreward as a series of large amplitude internal waves (Chereskin, 1983). The slicks are surface manifestations of currents generated over the tops of these internal waves (Ewing, 1950) and they delineate a zone of converging and downwelling currents situated over the trough of the waves (Fig. 7 in Zeldis and Jillett, 1982). As the internal waves travel shoreward the wave-generated currents sweep oil and flotsam into the convergence forming a slick (Ewing, 1950). Buoyant flotsam will remain at the surface trapped in the convergence and be carried along with the waves (Arthur, 1954; Shanks, 1983). The proposed mechanism of larval transport suggests that any organism which can maintain itself at the surface in the face of downwelling currents at the convergence will, like the flotsam, remain in the slick and be transported ashore (Shanks, 1983).

The purpose of this research was to investigate the mechanism of onshore transport of barnacle larvae. Onshore transport was not directly investigated, but was inferred from correlations of daily settlement of cyprids in the intertidal zone with physical factors in the environment which cause onshore flow:winds and tides. For daily settlement to be related to onshore transport cyprids must settle immediately upon reaching the shore and there must be an offshore reservoir of larvae which can be periodically swept onshore. Gaines *et al.* (1985) found that the abundance of planktonic *Balanus glandula* cyprids in the waters above an intertidal zone decreased dramatically with proximity to the shore while the abundance of similarly sized copepods did not decrease shoreward. They interpret this decrease in planktonic cyprids as due to their settling out upon contacting the bottom as they were swept through the intertidal. The planktonic stage in the life cycle of barnacles has received little attention and there are few published descriptions of their offshore distribution. Crisp and Southward (1958) and Kendell *et al.* (1985) both found an abundance of cyprids out to about 10 km off the coast of England and I have made similar observations in the Southern California and South Atlantic Bights (pers. obs.). These studies suggest that there is an offshore reservoir of cyprids and that cyprids settle soon after reaching the shore. Hence, the assumption that the pattern of daily settlement of cyprids is related to the forces carrying the cyprids onshore seems reasonable.

MATERIALS AND METHODS

Barnacle settlement was studied in the intertidal zone 1.5 km north of the Scripps Institution of Oceanography pier (35°5′N, 177°5′W). Winter and spring storms remove sand from this stretch of beach leaving a boulder/cobble field resting on a smooth shelf of sandstone. Sand accumulates during the late spring and summer until just the larger boulders protrude above the surface of the beach. From about 0 to 1.6 m above mean lower low water (MLLW) the dominant fauna on the boulders is the barnacle *Chthamalus* spp., which carpets many boulders almost completely. The tides are semidiurnal with a maximum range of nearly 3 m. The shore is protected from ocean swells by refraction of waves away from the study site due to a submarine canyon located immediately offshore (the Scripps Canyon). Maximum significant wave height (*i.e.*, average of larger one-third of the waves) measured at the Scripps pier was 1 m or less during the study.

Barnacle settling plates initially consisted of pancakes of Splash Zone® epoxy glued to the seaward face of large boulders located at +1.5, +1.0, and +0.3 m MLLW (Rocks 1, 2, and 3 respectively). Into each pancake three circular grooves (4 cm dia × 0.5 cm wide, surface area per groove 3 cm^2) were pressed. During a second period of observations a piece of plain ceramic floor tile was glued with the grooved bottom side up (3 grooves, 5 cm long × 0.5 cm wide, surface area per groove 2.5 cm^2) on the seaward side of Rock 2 (+1.0 m MLLW).

Counts were made daily between 9 April and 30 June, 1983, and 17 August and 1 November, 1983, during daytime low tides. Observations of settlement on Rock 3 stopped on 18 May (after 41 days) because the rock was buried by sand. During the second observation period settling was only followed at Rock 2. With the aid of 10× hand lens counts were made of the cyprids and newly metamorphosed barnacles present in the grooves on the settling plates. Barnacles were not identified to species, but given the extreme dominance of the boulders by *Chthamalus* spp. most of the settlers were probably *Chthamalus*. Following each daily count the grooves were vigorously brushed with a coarse brush which removed all visible (as viewed with a 10× hand lens) remains of cyprids and barnacles.

Cross correlations using the methods of Blackman and Tukey (1959) were calculated between the daily barnacle settlement and both wind direction and maximum daily tidal range. Wind direction data were taken from the records of the National Weather Service station at Lindberg Field, San Diego, California. Wind direction measured at this location is usually not different from that observed at Scripps pier (C. Winant, pers. comm.). Tidal range was taken as the maximum daily difference between a high tide and the next low tide during a 24 h period beginning at 18:00 h. Calculations of cross correlations were limited to lags of ±10% of the record length (Otnes and Enochson, 1978).

Before calculating the cross correlations, daily barnacle settlement was scaled to the hours of submersion at each tidal height. The purpose of these calculations was to control for variation in the duration of submersion over the semilunar tidal cycle that may have effected settlement in addition to semilunar tidally driven currents. The rate of barnacle settling steadily decreased during each observation period. Therefore the data were also detrended prior to the calculation of the cross correlations. Detrending (Otnes and Enochson, 1978) was accomplished as follows: a linear regression was calculated between the log of the barnacle settlement/h submersion/day and sampling sequence (*e.g.*, time); then the residuals from each day's datum were subtracted from the overall average daily settling rate (log of barnacle settlement/h of submersion/day) during the period of observation to give the detrended values.

RESULTS

During both sampling periods and at all tidal heights there was a clear rhythm to the daily abundance of settlers and this rhythm appeared to be related to the maximum daily tidal range (Figs. 1, 2). The period separating maximum barnacle settlement (starred data points in Figs. 1, 2) was not different from the period separating the maximum spring tide (Table I). Moreover, during the first sampling period the time separating maximum spring tides alternated between about 12 and 17 days as did the time between maximum barnacle settlement.

Inspection of Figures 1 and 2 suggests that peak settlement usually occurred one to several days before the spring tide. The results of the cross correlation calculations support this impression (Figs. 3, 4). Significant ($P < 0.01$) cross correlations occurred around +1 and +4 days lag. That is, maximum barnacle settlement occurred 1 to 4

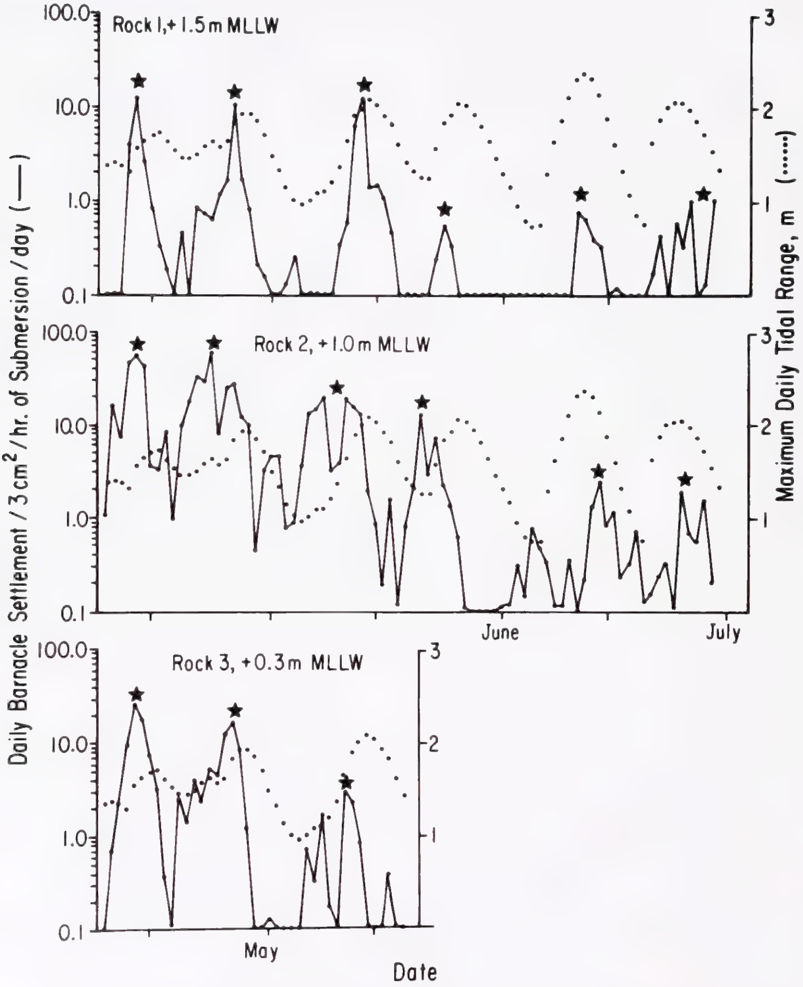

FIGURE 1. Daily barnacle settling rate (number/3 cm^2/h of submersion/day, solid line) at three tidal heights (+1.5, +1.0, and +0.3 m MLLW) plotted with the maximum daily tidal range in meters (dotted line). Data were collected from 9 April to 30 June, 1983, at Dike Rock, California. Stars over the plot of daily barnacle settling rate indicate dates of peak settlement. The small increase in settling rate which occurred around 5 June was not considered to be a peak in settlement because it was a tenth the size of the preceding peaks and only a third the size of the following two peaks.

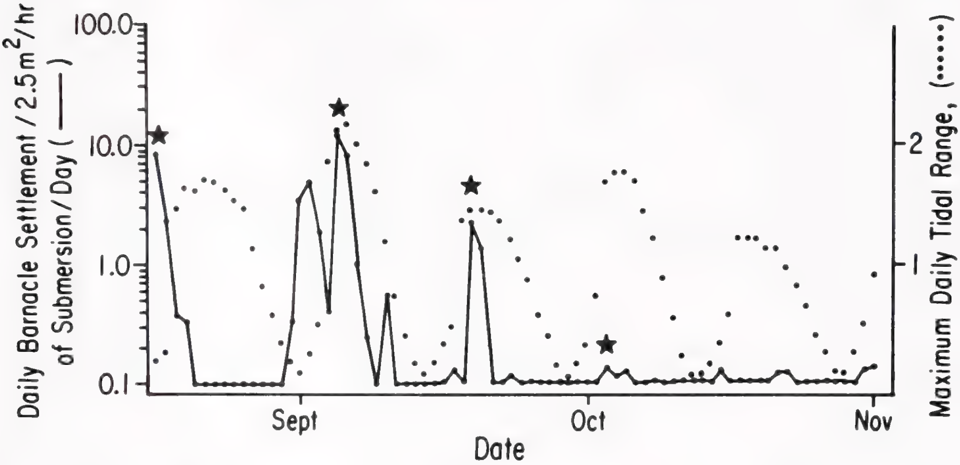

FIGURE 2. Daily barnacle settling rate (number/2.5 cm^2/h of submersion/day, solid line) at +1.0 m MLLW plotted with the maximum daily tidal range in meters (dotted line). Data were collected from 17 August to 1 November, 1983, at Dike Rock, California. Stars over the plot of daily barnacle settling rate indicate dates of peak settlement.

days before the maximum spring tide. From about 12% (Fig. 3, Rock 2) to 40% (Fig. 3, Rock 3) of the variation in barnacle settlement might be attributable to effects of the semilunar tidal cycle. Scaling the daily barnacle settlement by hours of submersion may have injected a tidal periodicity into the data. However, the detrended, but not scaled data (*i.e.*, number/day) also displayed similar significant cross correlations between settlement rate and the daily tidal range.

Through May in the first set of observations, maximum settlement at Rocks 1 and 2 occurred between the neap and spring tides. Around the end of May the timing of the peak settlement appears to shift such that it occurred at or just after the spring

TABLE I

Comparison of the period in days separating maximum spring tide and peaks in daily barnacle settling rate

	Period (days) between maximum spring tide and maximum barnacle settlement[1]					
	First sampling period (9 April–30 June, 1983)				Second sampling period (17 Aug.–1 Nov., 1983)	
Period	Tide	Rock 1	Rock 2	Rock 3	Tide	Rock 2
1	11	13	10	13	15	17
2	17	17	16.5	15	14	14
3	12	11	12.5	—	14	14
4	17	18	24	—		
5	12	16.5	11	—		
Mean	13.8	15.1	14.8	14.0	14.3	15.0
SE	1.3	1.3	2.6	1.0	0.3	1.0

[1] Days of peak barnacle settlement are labeled with stars in Figures 1 and 2.

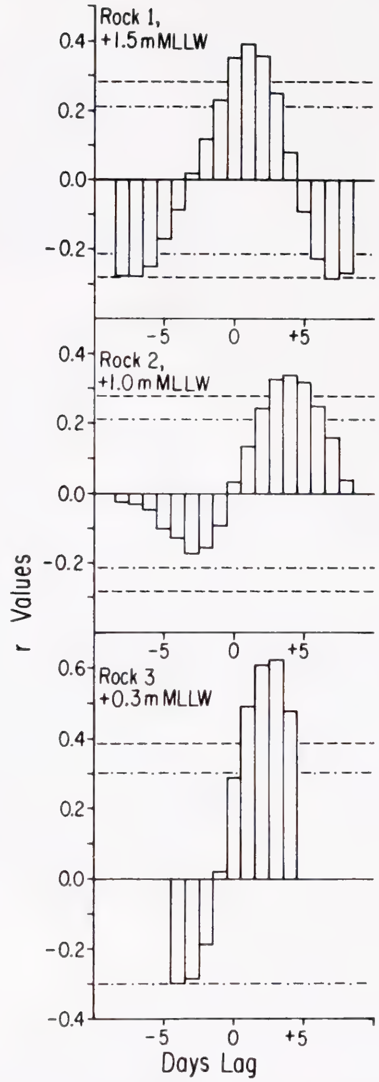

FIGURE 3. Cross correlations at three tidal heights (+1.5, +1.0, and +0.3 m MLLW) of the detrended daily barnacle settlement (Log (X + 1)/3 cm^2/h of submersion/day) *versus* the maximum daily tidal range. Data were collected over the period 9 April to 30 June, 1983. Cross correlation was limited to ±10% of the length of the time series (Otnes and Enochson, 1978). Critical $r_{0.05}$ (–·–) and $r_{0.01}$ (– – –) are also plotted.

tides (Fig. 1). There was also a sharp decrease in the rate of settlement which occurred at this time. This apparent shift was associated with a sharp drop in the sea surface temperature from about 18.5°C to about 14.5°C (Fig. 5).

There was no obvious correlation between wind direction and the daily rate of barnacle settlement (for example, Fig. 6). There were no significant cross correlations between the rate of barnacle settlement and either the resultant wind direction for an entire day (*i.e.*, the vector sum of wind speeds and directions measured every 3 h divided by the number of observation) or the wind direction during high tides. Set-

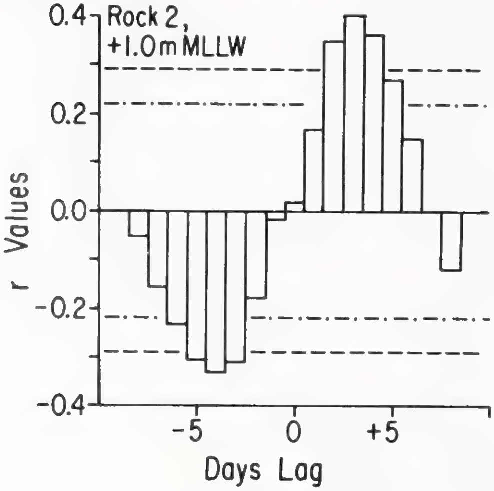

FIGURE 4. Cross correlation at +1.0 m MLLW of the detrended daily barnacle settlement (Log (X + 1)/2.5 cm^2/h of submersion/day) vs the maximum daily tidal range. Data were collected over the period 17 August to 1 November, 1983. Cross correlation was limited to ±10% of the record length (Otnes and Enochson, 1978). Critical $r_{0.05}$ (–•–) and $r_{0.01}$ (– – –) are also plotted.

tlement was not significantly different (*t*-test, $P > .10$) on days with onshore winds (winds from about 204 to 280°) than days with longshore winds. Resultant winds blowing offshore (winds from around 90°) did not occur during either observation period nor were there strong near shore winds.

DISCUSSION

At all three tidal heights and during both observation periods the daily settlement of barnacles was significantly cross correlated with the maximum daily tidal range. Maximum settling tended to occur one to four days before the spring tide. This relationship between daily settling rate and the semilunar tidal cycle is almost identical to that observed for settling of *Pachygrapsus crassipes* megalopae (Shanks, 1983, 1985b). Peak catches of these megalopae also tended to occur four days before the spring tide. The megalopae of *P. crassipes* are a larval form which is transported onshore in slicks over tidally forced internal waves (Shanks, 1983, 1985a). The similarity between the fortnightly cycle of barnacle settling and that of the *P. crassipes* megalopae suggests that the barnacle cyprids settling in the Dike Rock intertidal may also be carried ashore by internal waves.

On the southern California continental shelf the internal waves have a fortnightly cycle related to tidal range (Cairns, 1967, 1968). The largest waves, generated during spring tides, break and progress across the shelf as internal bores, producing near-bottom onshore flow and surface offshore flow (Winant and Olson, 1976; Winant and Bratkovish, 1981). The internal bores also produce turbulence which disrupt and weaken the thermocline on which subsequent internal waves propagate (Cairns, 1968). During tides with smaller range, trains of internal waves of lesser magnitude are formed which propagate across the shelf into shallow water (Cairns, 1968; Winant, 1974). If cyprids were using broken internal waves as a means of shoreward transport, one would expect maximum settlement around the spring tide when internal bores are present. But peak settlement consistently occurred several days before spring tide. This

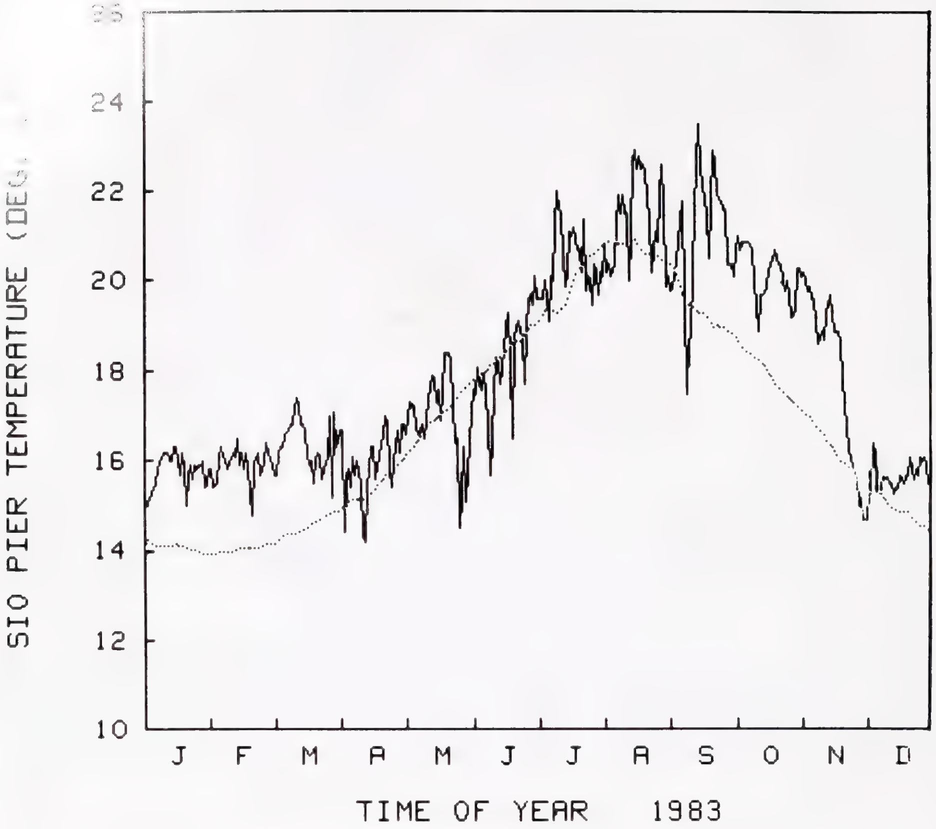

FIGURE 5. The daily sea surface temperature for 1983 (solid line) measured 1.5 km from the Dike Rock study area at the Scripps Institution of Oceanography pier. The dotted line is the 62 year mean sea surface temperature.

suggests that these cyprids may be transported onshore by the unbroken internal waves. High settlement of cyprids and megalopae did not occur during the periods between the spring and neap tides, periods when smaller internal waves ought to have been formed. The data presented in Shanks (1983) indicate that only some sets of internal waves cause onshore transport. Perhaps those internal waves produced during the periods between the spring and neap tides, due to the weakened thermocline, do not cause onshore transport.

A variety of physical factors effect the formation of these tidally generated internal waves (*e.g.*, tidal range, type of bottom topography, and water column density structure). The waters offshore of Dike Rock are characterized by mesotides (tidal range 2–4 m, Davies, 1964), a submarine canyon, narrow shelf (<3 km), and seasonally shallow thermocline (Cairns and Nelson, 1970). While onshore transport of larvae by internal waves occurs on other coasts (*e.g.*, New Zealand, Kingsford and Choat, in press; the South Atlantic Bight, pers. obs.) the pattern of daily settlement need not be similar to that observed at Dike Rock. This is because the physical environment in which the waves are formed will vary from site to site. My preliminary observations in the South Atlantic Bight, an area of microtides (tidal range < 2 m), suggest that

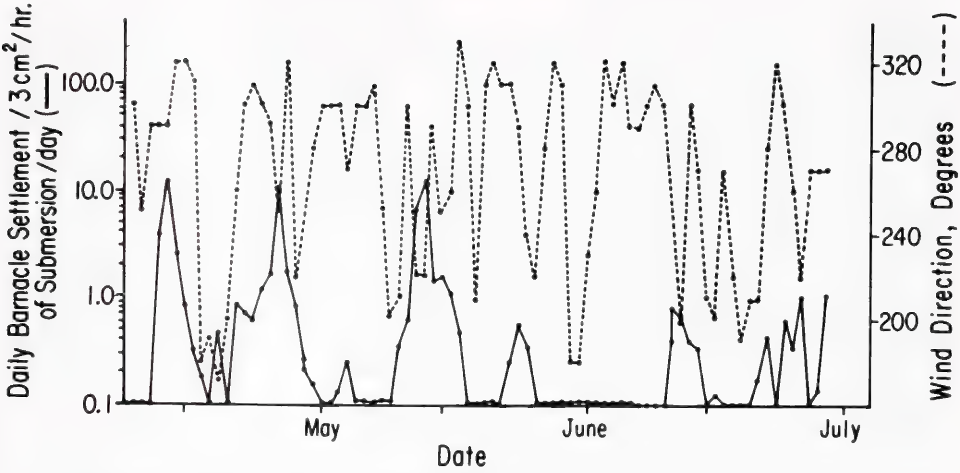

FIGURE 6. Daily barnacle settling rate (number/3 cm^2/h of submersion/day, solid line) at +1.5 m MLLW plotted with the daily resultant wind direction (dashed line). Wind direction is the direction from which the wind is blowing. Onshore winds come from around 270°. Settlement data were collected from 9 April to 30 June, 1983, at Dike Rock, California.

onshore-transporting internal waves are only formed around the spring tide and I would predict that settlement of organisms transported by these waves would be highest during these periods.

For an organism to utilize slicks over internal waves as a mechanism of onshore transport, it is necessary for it to remain at or near the surface. The megalopae of *P. crassipes* display a set of behaviors—positive phototaxis, negative geotaxis, high baro- and photokinesis, high swimming speed (9 cm/s), and high thigmokinesis (Shanks, 1985a)—which aid them in locating and remaining at the ocean's surface. The behaviors associated with the planktonic existence of cyprids have not been well studied but the behaviors which have been observed—positive phototaxis (Knight-Jones, 1953; Crisp, 1955; Crisp and Ritz, 1973), fairly high swimming speeds (4 to 5 cm/s, Crisp, 1955), and high barokinesis (Knight-Jones and Morgan, 1966)—are all behaviors which may, as they do for *P. crassipes* megalopae, aid cyprids in locating and remaining in the surface waters where transport by slicks can occur. In addition, the cyprids of some species are hydrophobic and may remain at the ocean's surface by sticking to the surface film (Knight-Jones, 1953; Crisp, 1955; Connell, 1956). However, actual contact of an organism with the ocean's surface is not necessary for internal-wave mediated transport to occur. The only available data on the vertical distribution of cyprids in the water column are those of De Wolf (1973) and Grosberg (1982). De Wolf (1973) found little vertical stratification of cyprids in a tidally well mixed estuary while Grosberg (1982), who collected samples in a small, poorly mixed harbor, found that cyprids of *Balanus glandula* were concentrated at the water surface (<0.5 m depth) while the cyprids of *B. crenatus* lived somewhat deeper (>2 m depth). I know of no published data on the vertical distribution of cyprids in offshore waters.

The observed correlation of settling rate with the tides might be due to the timing of adult spawning coupled with the rate of larval development. If, as has been observed in some crab populations (Christy, 1982), adult barnacles spawn on a fortnightly tidally timed cycle and the subsequent larval development is around two weeks, or some multiple thereof, then a similar cycle of larval settling in the intertidal might be observed. Even if adult *Chthamalus* spp. do spawn on a rigid fortnightly schedule (there is no evidence for or against this) larval development time is probably so variable as to mask the effect of adult spawning sychrony by the time the larvae are ready to settle. Development time has not been measured in *Chthamalus* spp., but it has for *Balanus glandula,* a species which occupies the same habitat and range as *Chthamalus* spp. (Morris *et al.,* 1980). Strathmann *et al.* (1981) found development times from release to cyprid ranging from 10 days at 17°C to 22 days at 9.5°C while cyprids could delay metamorphosis for from 19 to 35 days at temperatures of 17 and 9.5°C, respectively. During the first series of observations the temperature at the Scripps Institution of Oceanography pier varied from 18.5 to 14°C and during the second set from 22.5 to 18.5°C (Fig. 5). Despite these temperature variations, the period separating peaks in larval settlement was not significantly different from the period separating spring tides (Table I). Thus, it would seem that the duration of larval development is too variable to support the hypothesis that the observed discrete fornightly settlement cycle simply reflects the cycle of adult spawning.

Although the possibility that the daily rate of settling of barnacles is related to the semilunar tidal cycle has not previously been reported there are some data in the literature which support this observation. Wethey (1985) followed daily settlement of *Semibalanus balanoides* during two different settling seasons on the coast of Massachusetts. He found no relationship between daily settlement and wind direction, but he did note an apparent lunar periodicity to settling which might, in fact, be related to the semilunar tidal cycle. Peak settlement tended to occur several days before and after spring tides. Both Wethey (1985) and Kendall *et al.* (1982) followed daily settlement at intertidal stations along the Yorkshire coast, U.K. In Wethey's observations at three sites (Staithes, Robin Hood's Bay, and Filey) there is no obvious tidal periodicity to settlement, but in Kendall *et al.* (1982) daily settlement at Robin Hood's Bay (Fig. 2, Kendall *et al.,* 1982) does appear to vary on a tidal cycle: peak settling rates occurred between the neap and spring tides as was observed in this study. These studies suggest that in other areas daily barnacle settlement may be related to the semilunar tidal cycle.

Two studies, both from the Irish Sea, present data which suggest that daily settling rate was related to wind direction. In Hawkins and Hartnoll (1982) peak settling in 1979 occurred during onshore winds, while Bennell (1981), during the same 1979 season, suggests that peak settling rate occurred during offshore winds. There was no apparent tidal periodicity to the settling rate in either study. If cyprids of intertidal barnacles are carried onshore by tidally driven internal waves then perhaps the extreme tidal mixing which occurs over much of the Irish Sea (an area of macrotides, >4 m) and the complex distribution of marine fronts at either end of the sea (Simpson and Hunter, 1974; Simpson and Pingree, 1978) prevent the propagation of internal waves through the Irish Sea. In the absence of internal waves, the effects of wind direction on settling rate may then become apparent.

Additional data that bear on the mechanism of onshore transport are hard to come by. Attempts to correlate previous records of daily barnacle settling (Bennell, 1981; Hawkins and Hartnoll, 1982; Kendell *et al.,* 1982; Wethey, 1985) with either wind direction or tidal range are hampered by the shortness of the time series. These studies followed the settling of *Semibalanus balanoides,* a species with a brief springtime settling season. The longest record (Hawkins and Hartnoll, 1982) is only 60 days and the average published record is only 30 days. Correlations between such short time series and wind direction are probably acceptable. Wind events are usually only several days in duration (for example see Fig. 6), and, there might be 10 or more wind events during a month-long time series against which settling rate can be compared. For correlation against the semi-lunar tidal cycle the records are usually too short. If peak settling rates occur between the neap and spring tides, as was observed in this study, then the average time series will only contain two such periods. In this situation, cycles of tidal range and settling rate must be closely related (see for example Rock 3 this study where the time series is only 42 days) if significant correlations are to be found. If a species with a longer settling season had been studied perhaps a tidal periodicity to settling rate would be a more common observation.

The data presented in this paper suggest an hypothesis to explain the onshore migration of cyprids; they are transported onshore in slicks over tidally forced internal waves. From this hypothesis several predictions can be made and used to test the hypothesis. If cyprids, or any other larvae, are transported onshore in internal-wave slicks, then they must inhabit the surface waters. Further, if the hypothesis is correct, then in the waters over those internal waves which are causing onshore transport of surface drifters (Shanks, 1983), cyprids should be most abundant in the internal-wave slicks. Finally, if internal waves are 'catching' cyprids and carrying them shoreward, then there should be significantly fewer cyprids at the surface immediately behind a set of internal waves than immediately in front of the set.

ACKNOWLEDGMENTS

This study would not have been possible but for the help of my friends and colleagues William G. Wright and Waldo W. Wakefield. The temperature data for Figure 5 were provided by E. Stewart. The research greatly benefited from the advice of Dr. James Enright. The comments of Drs. William G. Wright, Richard R. Strathmann, and two anonymous reviewers on the manuscript are appreciated. I would especially like to thank my wife, Laurie, for so patiently tolerating a summer spent tramping out to the intertidal to collect the day's sample. The work was supported by the Marine Life Research Group of the Scripps Institution of Oceanography, University of California, San Diego.

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SURFACE TOPOGRAPHY INFLUENCES COMPETITIVE HIERARCHIES ON MARINE HARD SUBSTRATA: A FIELD EXPERIMENT

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ABSTRACT

Among colonial encrusting organisms, competitive relations often are intransitive, with individuals of subordinate species occasionally winning encounters with higher ranking species. Many mechanisms, including allelopathy, growth form, and disturbance have been proposed to explain this phenomenon. We show experimentally that such reversals of rank can occur purely as a result of surface topography. The bryozoan *Alcyonidium* was superior to the bryozoan *Electra* when encounters occurred on level substrata (8 wins, 0 losses). When *Electra* was given a 1.6 mm height advantage in the zone of contact, it won in 15 out of 16 encounters with *Alcyonidium*. This mechanism may explain the higher incidence of intransitivity among epifauna on the topographically variable *Fucus serratus* in Wales, compared to the competitive hierarchy seen on the topographically more uniform *Fucus distichus* ssp. *evanescens* and *Chondrus crispus* in New England.

INTRODUCTION

On space-limited natural and artificial marine hard substrata, interference competition via overgrowth is common (*e.g.*, Jackson and Buss, 1975; Jackson, 1979; Paine, 1984; Sebens, 1986). In most pairwise interspecific interactions, one species more frequently overgrows the other (*i.e.*, species A > species B in greater than 50% of the interactions). When pairwise interactions among all species are taken together—especially when different phyla are compared—the ranking of competitors appears hierarchical and mathematically transitive (A > B > C and A > C). In the absence of predation or disturbance, the rate at which the competitively dominant species eliminates subordinate species depends upon: (1) the frequency of interspecific contacts, (2) species-specific rates of recruitment into the system, (3) relative growth rates of species, and (4) the degree of asymmetry in interspecific competitive outcomes (*i.e.*, species A may not always overgrow species B). The less the certainty that species A will overgrow species B in a given interaction (and the greater the possibility of a competitive reversal in which species B actually overgrows species A), the slower the rate at which species A will monopolize space.

Although some authors consider reversals to be intransitivities (*e.g.* A > B > C and C > A) averaged over all interactions among three or more species, here reversals will be defined in terms of individuals. A reversal will be defined as any instance in which an individual of a subordinate species overgrows an individual of a dominant species. Using this definition, reversals play a small role in the maintenance of diversity if they are rare, but if they occur frequently, then they may be very important.

A variety of mechanisms have been proposed to explain competitive reversals in colonial organisms in the intertidal and subtidal. These include differences in the size

Received 21 May 1985; accepted 25 March 1986.

of individual combatting colonies (Day, 1977; Buss, 1980, 1981; Russ, 1982), encounter angle between colonies (Jackson, 1979; Buss, 1981; Rubin, 1982), allelochemical interactions (Jackson and Buss, 1975; Green, 1977; Jackson, 1979; Stoecker, 1980), differences in the growth form of competitors (Jackson, 1979; Buss, 1980, 1981), the influence of grazers and/or disturbance agents (Paine, 1984), and surface topography (Paine, 1984; Connell and Keough, 1985).

Three mechanisms involving vertical relief influence the outcomes of competitive interactions in epifaunal systems. First, the thicker of the two colonies usually wins (Buss, 1980; Seed and O'Connor, 1981; Russ, 1982; Sebens, 1985, 1986). This has been demonstrated in many communities with thick, fleshy organisms, such as the bryozoan *Flustrellidra* or the ascidian *Botryllus,* overgrowing much thinner species, such as *Electra* (LJW, per. obs.). Colony thickness, hence competitive ability, may not be a fixed species-specific trait, but dependent on the colony's age (Buss, 1980, 1981). Buss (1981) showed how one tropical cheilostome bryozoan species became thicker as it grew larger, thereby enhancing its competitive ability. Second, colonies may be able to elevate their growing edges over competitors thereby blocking overgrowth or overgrowing the competitor themselves (*e.g.,* frontally budding bryozoans such as *Schizoporella*) (Jackson and Buss, 1975; Buss and Jackson, 1979; Jackson, 1979). Third, the colonies occupying topographic highs may have a competitive advantage. Paine (1984) noted that certain coralline algal species, such as *Lithophyllum,* tend to occupy topographic highs, rendering overgrowth more difficult. Species located on the high spots of a topographically heterogeneous substrata may also be at an initial competitive advantage (Connell and Keough, 1985).

The work presented here is an experimental test of the hypothesis that differences in the elevation of the growing edges of interacting colonies cause reversals in a competitive hierarchy in a temperate epifaunal community. The outcome of competitive interactions between two species was reversed by changing the colony elevation at the point of contact. These results are compared to the patterns of overgrowth in epifaunal species growing on algal substrata in various locations throughout the year.

MATERIALS AND METHODS

Live colonies of the ascidian *Botryllus schlosseri* and the bryozoans *Electra* and *Alcyonidium* were collected on algae and on strips of plastic suspended from buoys in Long Island Sound at the Yale Peabody Museum Field Station, Guilford, Connecticut (41°16'N, 72°44'W), from June to August, 1984. Small pieces of algae containing an entire bryozoan colony were glued onto 2.5 cm × 7.5 cm × 1.6 mm pieces of clear acrylic plastic using cyanoacrylate glue. Stocks of *Botryllus* were maintained in a running seawater tank. When needed, *Botryllus* zooids were cut from the parent colony through the tunic with a razor blade and placed on clear acrylic in petri dishes kept moist with damp paper towels for approximately six hours. During this time the zooids attached to the new substrata (Grosberg, per. com.). Colonies of both phyla were kept in the running seawater tank for several days to ensure their viability. Only colonies of *Botryllus* that had budded at least once and bryozoans that still had functioning lophophores were used. Colonies were then suspended two meters beneath a raft in the 25°C waters of Long Island Sound in clear plastic slide boxes, open on both sides. Experiments were run from 12 July to 8 September. After bryozoan colonies had grown off the edge of the algae and onto the slide, competitive bouts between all viable colonies were set up. Every effort was made to make sure the growing edges of the two colonies would meet (frontal encounter, *sensu* Jackson, 1979) and to use colonies of approximately the same area. Level encounters, with both competitors in the same plane, and raised edge encounters, with one slide glued (with cyanoacrylate glue) above the edge of another (1.6 mm height difference) were randomly prepared and run simultaneously from 21 July to 8 September (Fig. 2). They were suspended from the raft in open slide boxes and examined every two days. Colony overgrowth was scored as a win when one species overgrew another for at least a ten-day period. At this point the superior organism often occupied at least 70% of both slides.

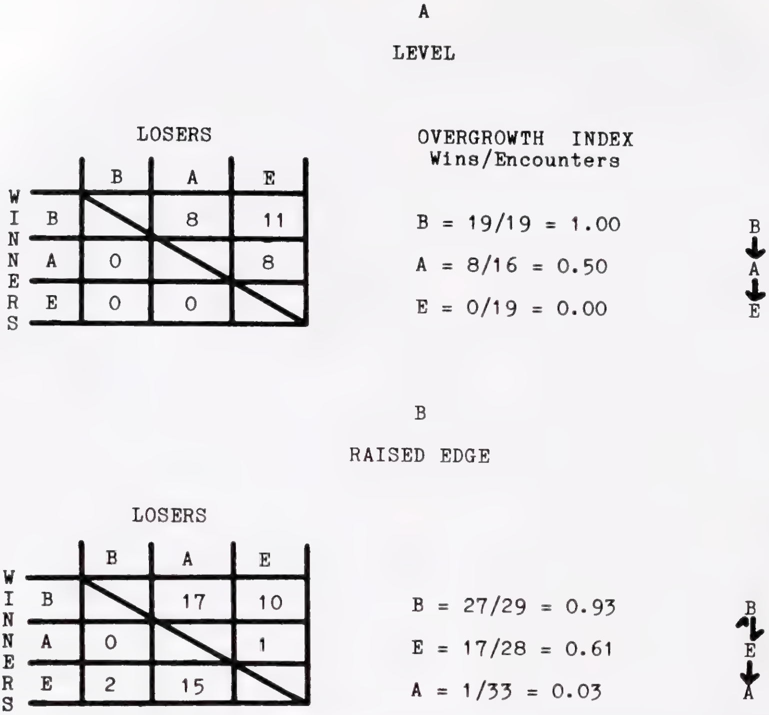
The outcomes of the experimental competitive interactions were compared to the pattern of interactions for common, encrusting, colonial species on natural algal substrata found in the low intertidal zone. The abundance, distribution, and overgrowths (defined as the occlusion of at least one zooid by the overgrowing colony, *e.g.*, Stebbing, 1973b) of epifauna were scored using a dissecting microscope. Measurements were made on *Fucus serratus* in North Wales in the spring of 1982, on *Fucus distichus* ssp. *evanescens* in northern New England (Harpswell, Maine, and Durham, New Hampshire) in the fall of 1982 and on *Chondrus crispus* on Horse Island in Long Island Sound during the summer of 1984 while the experiment was running. Although these measures are static and may not represent the final outcome of all pairwise competitive encounters (Ayling, 1983; Keough, 1983, 1984), hierarchies are apparent. All data were tabulated as win-loss matrices (*e.g.*, Jackson and Buss, 1975; Osman, 1977; Russ, 1982; Paine, 1984). An overgrowth index was calculated by dividing the number of wins for each species by the number of encounters in which the species was involved (Taylor, 1979; Rubin, 1982). The index is a measure of competitive ability. Diagrams show where hierarchies exist.

We define the "dominant" in the pairwise interactions to be the species that wins more than 50% of its encounters. "Consistency" of the outcome of a particular pairwise interaction is defined as the percentage of encounters in which the "dominant" species wins. "Reversals" of competitive outcomes, scored on an individual basis, thus contribute to reduced "consistency" (or greater "ambiguity") of outcomes on a population basis.

RESULTS

Field experiments

When species competed on level surfaces, outcomes were hierarchical (Fig. 1A). *Botryllus* overgrew *Alcyonidium* in eight out of eight encounters and *Electra* in 11 out of 11 encounters (Fig. 1A). In turn, *Alcyonidium* overgrew *Electra* in all cases (Fig. 1A), suggesting that competitive relationships between the three species are completely transitive (B > A > E & B > E). However, where *Electra* had a height advantage at the contact margin, it won 15 of 16 encounters with *Alcyonidium* (Fig. 1B). This is a reversal of ranks of *Electra* and *Alcyonidium*. This reversal of rank, dependent on elevation, is evident in the events that occurred on one of our plates (Fig. 2). *Electra* with a 1.6 mm height advantage overgrew the *Alcyonidium* beneath it (Fig. 2C, D). However, on the right hand side of the slide, *Alcyonidium* grew up onto the top slide and there on the level it overgrew *Electra*. It could be argued that the results are confounded in counting a level and an edge interaction for the same pair of colonies because feeding currents are shared and there may be a reaction to the interaction that occurred first. However, this is really the best test of the hypothesis, seeing the outcome reversed in the same colonies with a height change. Figures 2C, D, and E show a unique case in which a third competitor, a *Botryllus* colony, settled on the back of the slide, and grew around to the front. It then overgrew both *Electra* and *Alcyonidium* (Fig. 2C, E).



A

LEVEL

WINNERS \ LOSERS	B	A	E
B		8	11
A	0		8
E	0	0	

OVERGROWTH INDEX
Wins/Encounters

B = 19/19 = 1.00
A = 8/16 = 0.50
E = 0/19 = 0.00

B

RAISED EDGE

WINNERS \ LOSERS	B	A	E
B		17	10
A	0		1
E	2	15	

B = 27/29 = 0.93
E = 17/28 = 0.61
A = 1/33 = 0.03

FIGURE 1. The outcome of experimental competitive interactions. Numbers in the arrays show the overgrowths seen in pairwise competitive interactions for species on A: level clear plastic surfaces and B: plastic surfaces with a raised edge. Taxa involved include B—*Botryllus*, A—*Alcyonidium*, and E—*Electra*. The winners are listed in the rows of each table and the losers in the columns. For example, on the level, *Botryllus* beat *Alcyonidium* eight times and never lost to it. The second column shows the ratio of wins to total number of encounters for each species (overgrowth index). This provides an index of each species' competitive rank in that system. The diagramed competitive rankings show this ordering. If the overgrowth index in a particular pairwise interaction is greater than 10:1, single-headed arrows are used, pointing to the subordinate species. If the win/encounter ratio is less than 10:1, arrows are drawn in each direction proportional to the ratio.

On substrata where *Botryllus* was at a lower elevation than *Electra* or *Alcyonidium*, during initial frontal encounters, both *Electra* and *Alcyonidium* appeared to overgrow *Botryllus* for up to four days (Fig. 3D). Subsequently, *Botryllus* overgrew most competitors (25 of 27 interactions, Fig. 1B) by flanking the competitor on the higher plane (Fig. 3E, F). Within six days of the initial encounter, *Botryllus* overgrew most competitors from the sides. In all occurrences of intraspecific *Botryllus* interactions (11 level encounters, 5 raised edge encounters), the colonies apparently fused.

Natural algal substrata

Epifaunal coverage exceeded 90–100% of the area of some *Fucus serratus* in North Wales. A hierarchy of competitive ability appears to exist, although many ambiguities are seen in the outcomes of encounters (Fig. 4A). The consistency of competitive outcomes was varied from a high of 93% (between *A. polyoum* and *A. hirsutum*) to a low of 65% (between *Celleporella* and *Electra*. Overall, the consistency of outcomes was 81%. Fewer encrusting species are common in Maine and New Hampshire (Fig.

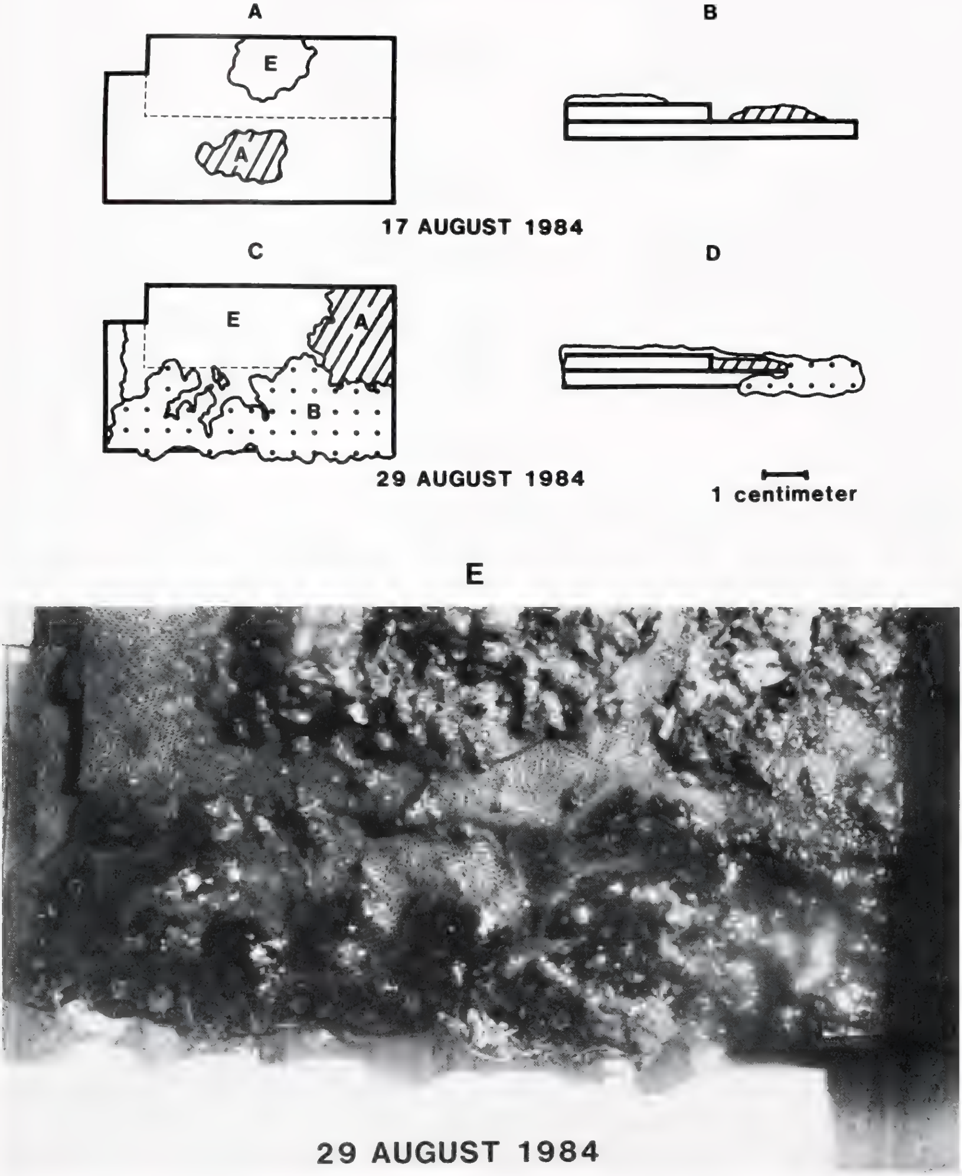


FIGURE 2. An example of a raised edge competitive interaction. Drawings were made by tracing photographs (taken with a Nikonos 35 mm camera fitted with a 2:1 extension ring) of the colonies' outlines on to tracing paper using a light table. In A and B, *Electra* (E) and *Alcyonidium* (A) are shown at the start of the experiment on August 17. In C and D, 12 days later, *Electra* with a 1.6 mm height advantage has grown over the *Alcyonidium* beneath it. However, a portion of the *Alcyonidium* colony escaped overgrowth long enough to invade the higher surface. On the level, *Alcyonidium* is overgrowing the *Electra* colony. A *Botryllus* colony (B), that settled on the back of the lower slide, has grown around to the front and is overgrowing both *Electra* and *Alcyonidium*. E is the photo from which C was taken.

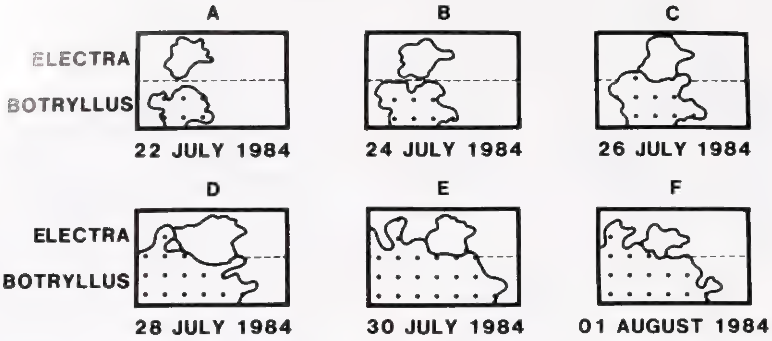


FIGURE 3. *Botryllus* defense when initially losing in a competitive bout with a height disadvantage. Long tunic arms grow over onto the higher slide. Within days it overgrows its competitor on the level at the flanks.

4B). A hierarchy is more apparent here and it is similar to that found in Long Island Sound (Fig. 4C). In Maine and New Hampshire, and in Long Island Sound, four out of five pairwise interactions had greater than 99% consistency of outcomes. Two of the species, *Electra* and *Membranipora,* rarely interact because their seasons of maximum abundance differ. In all observations on algal substrata, *Alcyonidium* dominates *Electra* (Fig. 4).

DISCUSSION

Although the outcomes of competitive interactions are often assumed to be independent of environmental conditions, a number of recent studies contradict this assumption. This study examined the influence of elevation differences on competitive rankings of species in an epifaunal community. Where colonies interacted on a level surface, a completely transitive competitive hierarchy resulted with *Botryllus* overgrowing both *Alcyonidium* and *Electra,* and *Alcyonidium* overgrowing *Electra.* Even where *Botryllus* was at an initial height disadvantage with respect to *Alcyonidium* and *Electra,* it continued to be the dominant overgrowth competitor by quickly flanking the other individual on the higher surface (Fig. 1B). In contrast, when *Electra* was elevated with respect to *Alcyonidium,* it overgrew *Alcyonidium* in 15 of 16 contacts (Fig. 1B). This suggests that colony elevation is an important determinant of the outcomes of encounters between encrusting bryozoans.

The results of this experiment contrast with those of coralline algae on topographically heterogenous substrata and smooth surfaces (Paine, 1984). On natural rough substrata with grazers, Paine (1984) found the outcomes of pairwise encounters to be 80% consistent; on natural rough surfaces with herbivores excluded, outcomes were 96% consistent; and on smooth, ungrazed surfaces, outcomes were 97% consistent. The results suggest that the presence of grazers increases the ambiguities in the outcomes of encounters on "natural" substrata. In *Lithothamnion,* for example, the ratio of wins to losses changed in the presence of grazers. The effects of grazing are unclear on smooth surfaces. In the absence of herbivory, topography introduces little ambiguity in the outcomes of interactions in these coralline algae. Paine (1984) notes however that survival of *Lithophyllum* is enhanced by occupying topographic highs, thus rendering its overgrowth by *Pseudolithophyllum* more difficult, suggesting that even in this system topography is important. In the present experiment, with no apparent predation, differences in colony elevation at the contact margin did cause reversals of

A

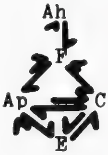
NORTH WALES

LOSERS

WINNERS	F	Ah	Ap	E	C
F		1	5	123	8
Ah	3		14	107	0
Ap	2	1		34	5
E	23	17	11		30
C	2	0	4	16	

OVERGROWTH INDEX
Wins/Encounters

Ah = 124/143 = 0.87
F = 137/167 = 0.82
Ap = 42/76 = 0.55
C = 22/65 = 0.34
E = 81/361 = 0.23



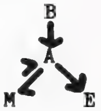
B

MAINE & NEW HAMPSHIRE

LOSERS

WINNERS	B	A	E	M
B		2	16	5
A	0		258	2
E	0	1		0
M	0	1	0	

B = 23/23 = 1.00
A = 260/264 = 0.99
M = 1/8 = 0.13
E = 1/275 = 0.00



C

CONNECTICUT

LOSERS

WINNERS	B	A	E	M
B		27	10	12
A	5		22	171
E	0	2		0
M	0	1	1	

B = 49/54 = 0.91
A = 198/228 = 0.87
E = 2/35 = 0.06
M = 2/185 = 0.01

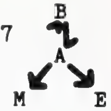


FIGURE 4. The outcome of competitive interactions on natural substrata. Numbers in the arrays show the overgrowths seen in pairwise competitive interactions for species on A: North Wales, B: Maine and New Hampshire and C: Long Island Sound. Species included are F—*Flustrellidra hispida*, Ap—*Alcyonidium polyoum*, Ah—*Alcyonidium hirsutum*, E—*Electra pilosa* and C—*Celleporella halina*, B—*Botryllus schlosseri*, A—*Alcyonidium* sp., and M—*Membranipora membranacea*. The notations used are the same as in Figure 1.

outcomes in competition. Either the two systems are substantially different or on Paine's (1984) natural, ungrazed surfaces, lower ranking species were not present on high spots.

Differences in topographic complexity may explain the lower consistency of outcomes of interspecific encounters and increased species diversity in the natural epifaunal assemblage in Wales *versus* the northeastern United States. In Wales, the epifauna are [illegible] on *Fucus serratus,* which has surface relief in the form of a thick midrib. The Connecticut data, which are more hierarchical, were collected on algae (*Fucus distichus* ssp. *evanescens* and *Chondrus crispus*) that have very little surface relief. Similarly, [illegible] and Day (1983) showed greater epifaunal species diversity on crinkled than on smooth *Laminaria* plants. Thus the comparison of topographically simple and complex habitats conforms to the predictions from our experiment.

If there are spatial refuges from competition, selection should favor larvae that preferentially settle in those refuges, or colonies that grow towards refuges after settlement (*e.g.,* Buss, 1979). Topographic highs are local temporary refugia from competition (*e.g.,* Fig. 1), but contrary to expectation, larvae apparently do not settle preferentially on them. Several species are rugophilic, but settle in depressions (not topographic highs) on crinkly algae or the troughs along the midrib of *Fucoid* algae (*e.g.,* Crisp and Williams, 1960; Eggleston, 1972; Ryland, 1976; Berstein and Jung, 1979). Settlement behavior and colony growth in relation to topography merit further study.

Alternative mechanisms that promote coexistence include growth form changes and avoidance of competitors at the time of larval settlement. The polychaete *Spirobis* can reorient its tube angle to grow above a competitor (Stebbing, 1973b). *Electra* has been shown to produce spines at the margin of contact which reduces the rate of overgrowth (Stebbing, 1973a). Several taxa of marine invertebrates avoid settling on substrata where there is a high probability of death from a superior spatial competitor (*e.g.,* Grosberg, 1981; Young and Chia, 1981).

A number of mechanisms (*e.g.,* density and size-dependent competitive ability, predation, physical disturbance, habitat selection, etc.) have been proposed to explain the maintenance of diversity in space-limited marine epibenthic communities. Any process which either decreases the likelihood of an encounter between a dominant and a subordinate species, or increases the likelihood of a normally subordinate species surviving (or even winning) such an encounter, should act to slow the rate at which diversity is lost from the system. Our results show that subordinate species may obtain an elevation-dependent competitive advantage or temporal refuge from competition in a topographically complex environment.

ACKNOWLEDGMENTS

This study was supported by The National Science Foundation (OCE 82-08176), the Office of Naval Research (contract N00014-82-K-0645) and the University of South Carolina. R. Seed and E. Sideman helped with the early stages of this work on natural substrata. L. W. Buss provided access to the field sites and lab space at the Yale University Peabody Museum Field Station. J. Sears assisted in taking photographs and M. Luckenbach helped in the darkroom. R. Grosberg, S. A. Woodin, R. Showman and two anonymous reviewers made many helpful comments on the manuscript.

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STRUCTURE OF THE NERVOUS SYSTEM OF THE AURICULARIA LARVA OF *PARASTICOPUS CALIFORNICUS*

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ABSTRACT

The structure and organization of the larval nervous system of the holothurian, *Parasticopus californicus,* is described using glyoxylic acid-induced fluorescence, indirect immunofluorescence with antibodies against serotonin, and transmission and scanning electron microscopy. Tracts of catecholaminergic axons are located at the base of the ciliary bands and catecholaminergic nerve cell bodies are dispersed along the length of the ciliary bands. Clusters of catecholaminergic cells form a ganglion on the lower lip of the larva and a ganglion of serotonergic cells is located at the anterior tip of the larva. Serotonergic cells are scattered throughout the apical portion of the larva in the epidermis. Axon tracts identified only with TEM are located in the esophagus associated with the circumesophageal muscles. The neuroanatomy of the auricularia shares several features with larval forms of the other classes of echinoderms.

INTRODUCTION

Holothurians typically have either planktotrophic development in which a feeding auricularia larva metamorphoses into a non-feeding doliolaria larva, or lecithotrophic development in which the embryo forms directly into a doliolaria (reviewed by Hyman, 1955). Forms which develop directly into the pentactula or juvenile without an intervening larval stage are also known. There are numerous accounts of the development of holothurians which describe the general features of larval structure (MacBride, 1914; Mortensen, 1931, 1937, 1938; Hyman, 1955; Inaba, 1957; Oguru, 1974). As well, Strathmann (1971) has given a comprehensive account of feeding in the auricularia and there are descriptions of metamorphosis and settlement of doliolariae (Ohshima, 1921; Inaba, 1957; Young and Chia, 1982). However, detailed knowledge of larval structure and the processes of metamorphosis for this class of echinoderms are fragmentary (reviewed by Hyman 1955, Strathmann, 1978).

The information available on other forms of echinoderm larvae, especially echinoids, is far greater. This is due in part to the difficulties encountered in obtaining gametes and raising holothurian embryos and larvae. Until recently techniques for obtaining fertilizable ova from most species of sea cucumber did not exist. Maruyama (1980, 1985), Kishimoto *et al.* (1982), and Smiley (pers. comm.) have developed techniques for the use of radial nerve extracts and disulphide reducing agents which permit detailed studies of holothurian larvae grown in culture (Maruyama, 1980).

Here we describe the organization and structure of the nervous system of an auricularia. Aspects of the neuroanatomy of four of the distinctive larval forms of echinoderms are now available, permitting a comparison of the general features of neural organization and the cytological characteristics of neural tissues of larval echinoderms.

Received 6 February 1986; accepted 24 March 1986.

MATERIALS AND METHODS

Larvae were reared using the techniques outlined by Strathmann (1968). Adults were collected subtidally in the vicinity of Friday Harbor, Washington, and Victoria, British Columbia. Gonadal tubules were dissected from females and put in seawater containing an extract of radial nerve prepared from *P. californicus* (the generous gift of Mr. Scott Smiley). After 1–2 hours germinal vesicles had begun to break down and eggs were expelled from the gonadal tubules. Eggs were transferred to culture dishes and a dilute suspension of sperm, prepared from dissected testes, was added. Water was changed every 24–48 hours and cultures fed a suspension of *Dunaliella salina*. Sixteen- to twenty-seven-day-old auriculariae were used for all experiments and observations. Larvae metamorphosed to doliolariae beginning about 25 days after fertilization and settled as pentactulae at about 30 days.

Specimens were prepared for glyoxylic acid-induced fluorescence following the procedures detailed in Burke and Gibson (1986). For immunofluorescence larvae were fixed in 4% paraformaldehyde in filtered seawater (FSW) for 2.5 h at room temperature and stored in FSW containing 0.01 *M* sodium azide. Fixed larvae were incubated in phosphate buffered saline (PBS) containing 10% goat serum and 0.3% Triton X-100 for 30 min to reduce non-specific binding and increase permeability. Specimens were incubated in rabbit anti-serotonin (diluted 1/90 in PBS) (Immuno Nuclear Corp.) for 16 h at 4°C, rinsed in PBS, and incubated in FITC conjugated goat anti-rabbit IgG (1/16 PBS) for 1 h at room temperature before being viewed and photographed with a Zeiss Universal microscope fitted for epifluorescence. The specificity of the primary antibody was assessed by pre-absorbing a 1/45 dilution of it with an equal volume of 1 mg/ml serotonin for 30 min.

Larvae were prepared for transmission electron microscopy (TEM) following the methods outlined in Burke (1985). Specimens for scanning electron microscopy were fixed in 2.5% glutaraldehyde buffered in 0.1 *M* sodium cacodylate, pH 7.3, to which several drops of 4% OsO_4 had been added. After 5 min in this solution larvae were transferred to buffered glutaraldehyde containing no OsO_4. After several months storage in this fixative larvae were rinsed well with 0.1 *M* sodium cacodylate and fixed with 1% OsO_4 in 1.25% $NaHCO_3$ for 1 hour before dehydration in a graded series of ethanol and critical point drying. Specimens were sputter coated with gold and viewed on a JEOL JSM-35 scanning electron microscope.

RESULTS

The auricularia larva of *P. californicus* is typical of holothurian auriculariae described previously (Mortensen, 1937, 1938; Strathmann, 1971). It has a single, sinuate circumoral ciliary band that is its principal feeding and locomotory organ (Fig. 1). The mouth, which is overhung by the pre-oral hood, is surrounded by a second ciliary band, the adoral ciliary band. Larvae used in this study, 16 to 27 days, increased slightly in size, developed more pronounced looping of the circumoral ciliary band, and elaborated the axohydrocoel and spherules, but otherwise changed little in form.

Ciliary bands in the auricularia are thickened regions of the epidermis with a row of densely packed, simple cilia. The bands range from 10 to 20 μm in thickness and typically have a number of evenly spaced mesenchyme cells associated with the blastocoelar surface (Fig. 2). The mesenchyme cells extend numerous filiform processes into the blastocoel.

In glyoxylic acid treated preparations, fluorescent tracts are associated with all of the ciliary bands of the auricularia. The fluorescent tracts are 2 to 3 μm thick and situated medially at the base of the ciliary band (Figs. 3, 4, 5). At intervals ranging

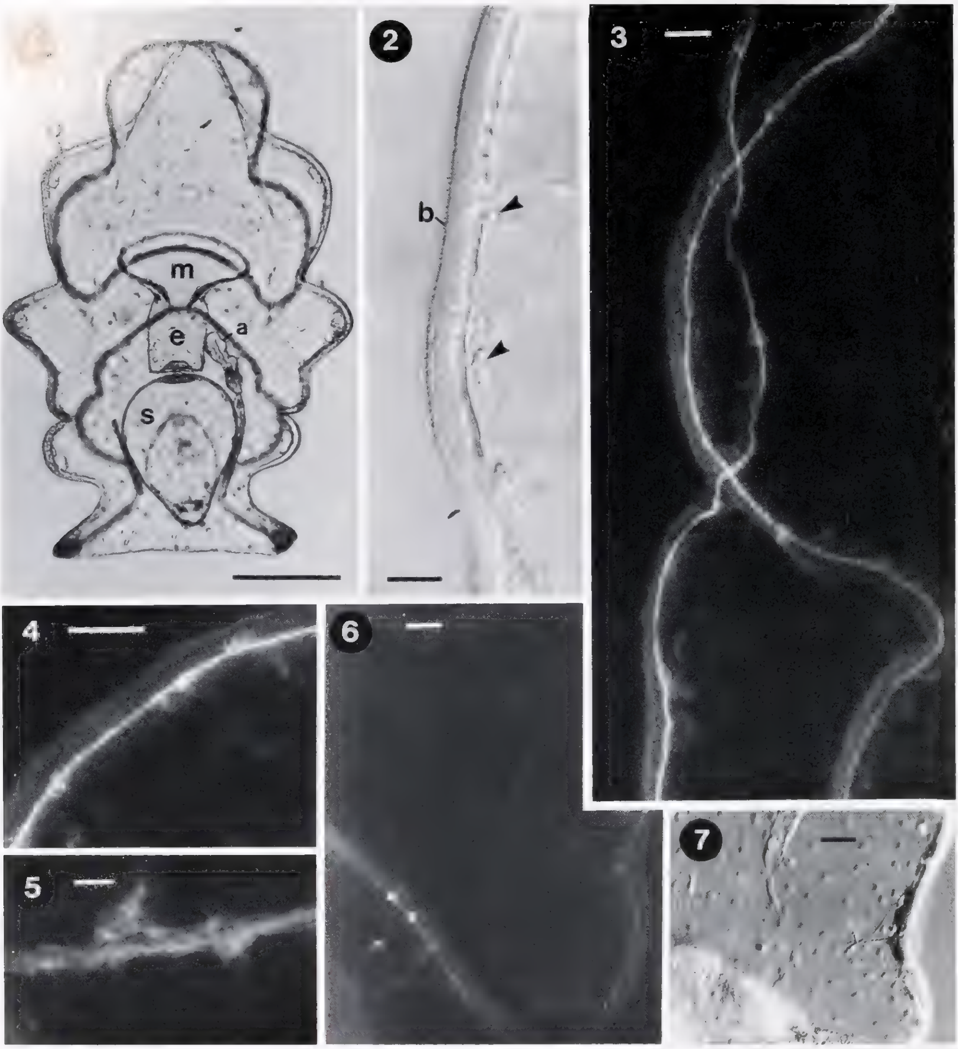


FIGURE 1. Auricularia larva of *Parasticopus californicus* 18 days after fertilization. a, axohydrocoel; b, circumoral ciliary band; e, esophagus; m, mouth; s, stomach. Bar = 100 μm.

FIGURE 2. Detail of circumoral ciliary band (b). Arrows indicate mesenchymal cells associated with the ciliary band. Nomarski differential contrast (DIC) optics. Bar = 20 μm.

FIGURE 3. Glyoxylic acid-induced fluorescence of catecholamines. A region of the circumoral ciliary band showing the fluorescent tracts situated at the base of the ciliary band. Bar = 20 μm.

FIGURE 4. Fluorescent tract and cell bodies associated with the circumoral ciliary band. Glyoxylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 5. Detail of fluorescent tract showing individual strands and cell bodies. Glyoxylic acid-induced fluorescence. Bar = 5 μm.

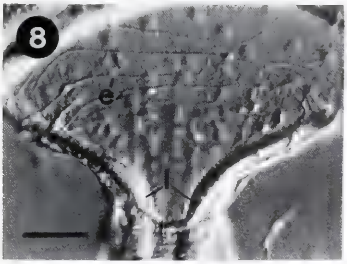
FIGURE 6. Fluorescent tracts and associated cell bodies that lie lateral to the larval mouth. Glyoxylic acid-induced fluorescence. Bar = 20 μm.

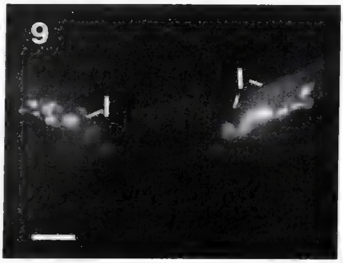
FIGURE 7. Triradiate tracts and cell bodies that lie lateral to the mouth in a live auricularia of *P. californicus*. Nomarski DIC optics. Bar = 20 μm.

from 50 to 150 μm along the length of the fluorescent tracts are 5 to 10 μm diameter thickenings that appear to be cell bodies (Figs. 4, 5). At higher magnification the fluorescent tracts can be resolved as numerous strands (Fig. 5). On both sides of the larva, lateral to the mouth, there are fluorescent tracts that are not associated with a ciliary band (Fig. 6). These tracts appear to join the lateral tracts associated with the pre-oral and post-oral ciliary bands. Often one or more cell bodies are associated with these branches. With Nomarski differential interference contrast optics, these bilaterally paired triradiate tracts and the cells associated with them can be resolved in live specimens (Fig. 7).

The mouth of the larva is an elliptical opening about 125 μm wide and 75 μm high in the middle of the oral field (Fig. 1). The adoral ciliary band is 6–7 μm thick around the perimeter of the mouth except in the notch that forms the lower lip where the band thickens to about 10 μm (Fig. 8). Fluorescent tracts occur throughout the adoral ciliary band of glyoxylic acid treated specimens in a similar location as described for those of the circumoral ciliary band. On the lower lip there is typically an aggregation of from 9 to 15 brightly fluorescent cell bodies (Figs. 9, 10). The cells are irregular in shape and up to 12 μm in diameter. Concentrations of cells are greatest on the sides of the notch of the lower lip and appear scattered up the adoral ciliary band.

At the apical tip of the larva the circumoral ciliary bands of the left and right sides approach each other but remain separated by 10 to 15 μm of epidermis. In glyoxylic acid preparations a fluorescent tract underlying the epidermis joins the fluorescent tracts of the ciliary bands (Fig. 11). Cell bodies were never observed in this region using this technique. However, in indirect immunofluorescent preparations using anti-serotonin antibodies, a ganglion of 10 to 12 cells was shown to lie beneath the epidermis at the apical tip of the larva (Figs. 12, 13). The cells are 10 to 12 μm in diameter, polygonal in outline, and multipolar (Fig. 13). The cells of the apical ganglion are interconnected by numerous axonal and dendritic processes up to 2 μm in diameter. As well, a number of immunoreactive cells that are similar in appearance are scattered along the apical circumoral ciliary band. No other immunoreactive cells were identified throughout the larval body. In control experiments in which the primary antibody was pre-absorbed with serotonin, fluorescence in these cells was reduced to nearly background levels.





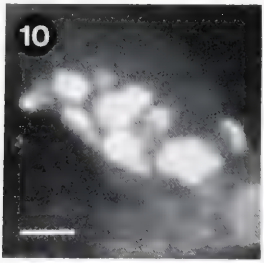
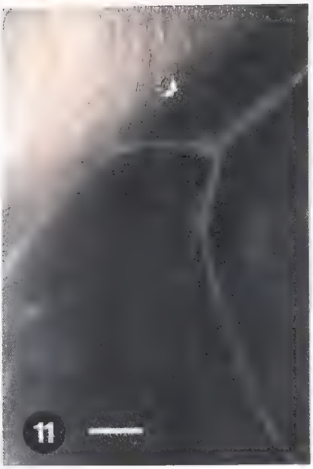
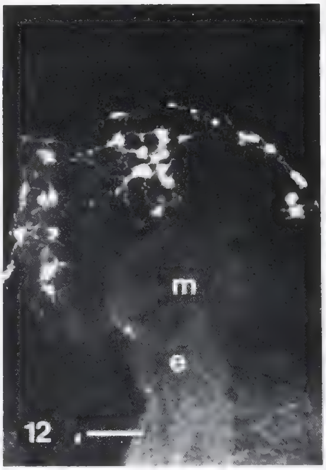


FIGURE 8. Thickened regions of the adoral ciliary band which form the lower lip (l) of the larval mouth of an auricularia of *Parasticopus californicus*. e, esophageal muscles. Nomarski DIC optics. Bar = 20 μm.

FIGURE 9. Fluorescent cell bodies within the lower lip (l). Gloxylylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 10. Detail of irregularly shaped cell bodies in the lower lip. Glyoxylic acid-induced fluorescence. Bar = 10 μm.





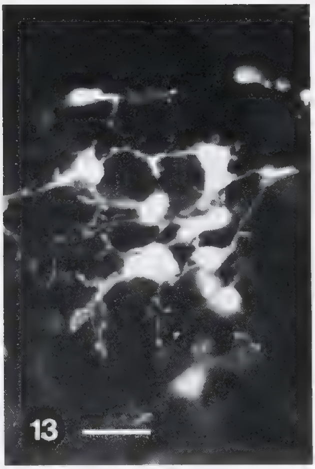


FIGURE 11. Fluorescent tract that joins the right and left circumoral ciliary band tracts at the apical tip of the auricularia larva of *Parasticopus californicus.* Glyoxylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 12. Indirect immunofluorescence with anti-serotonin showing the ganglion at the apical end of an auricularia larva. e, esophagus; m, mouth. Bar = 40 μm.

FIGURE 13. Detail of the multipolar cells and cell processes of the apical ganglion. Indirect immunofluorescence using anti-serotonin. Bar = 10 μm.

Ultrastructure

The thickened epithelium of the ciliary bands of the auricularia are comprised of numerous flask-shaped, ciliated cells with their tapered apices aggregated to form the densely ciliated band (Fig. 14, inset). The ciliated cells have a single cilium at their apical end, a centrally located nucleus, and contain various organelles including mitochondria, Golgi bodies, lysosomes, and axially oriented microtubules. The ciliated cells extend the full thickness of the band and their bases aggregate around a single tract of axons (Fig. 14). Occasionally in sections of the ciliary band, nerve cells extending axonal processes into the axonal tract can be identified. These cells typically have an elaborate, electron-dense Golgi body surrounded by numerous 0.1 to 0.4 μm diameter vesicles (Fig. 15). The nerve cells are about 12 μm in length and appear to extend to the outer surface of the larva. The axons range in size from 0.2 to 1.0 μm in diameter and indeterminate length (Figs. 16, 17). The number of axons within the tract varies from 8 to about 40. The clear cytoplasm of the axons contains numerous microtubules, mitochondria, and vesicles. Typically the vesicles range in size from 0.05 to 0.10 μm and contain either fine granular material or an electron-dense core. Vesicles of both types can be found within the same axon (Figs. 16, 17).

The adoral ciliary band on the lower lip can be seen in scanning electron micrographs to form two ciliated palp-like prominences (Fig. 18). In section, the palps are a simple columnar epithelium made up of ciliated cells, interspersed with nerve cells (Fig. 19). At the base of the epithelium are numerous axons aggregated into tracts that extend the length of the esophagus (Fig. 20). Nerve cells of the adoral ciliary band are elliptical in outline and are distinguished by a prominent, centrally located Golgi body which is surrounded by numerous 0.05 to 0.1 μm vesicles. The apical half of these cells contain 0.2 to 0.6 μm vacuoles which are irregular in outline and contain electron-dense material condensed into a loose irregular meshwork (Fig. 21). Throughout its

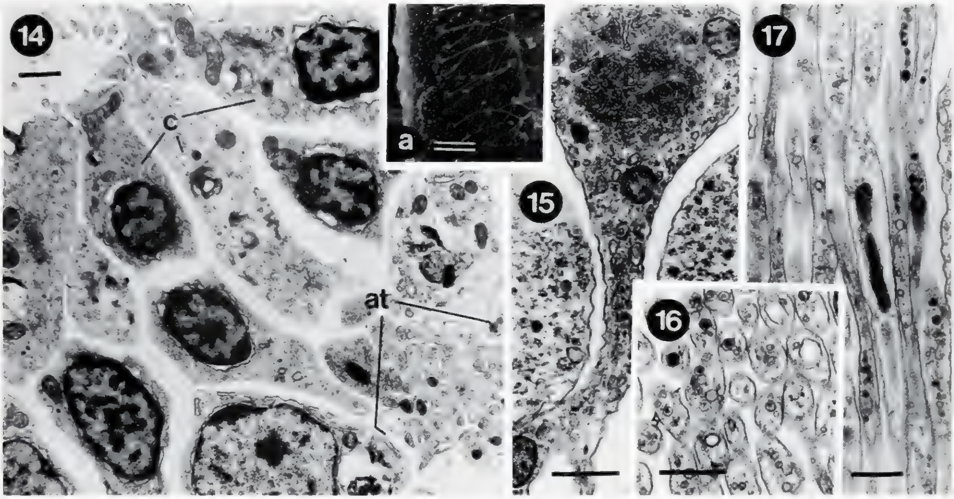


FIGURE 14. Transmission electron micrograph (TEM) of a transverse section through the circumoral ciliary band of a 23-day-old auricularia showing the ciliated cells (c) and the tract of axons (at) that lies at the base of the ciliated cells. Bar = 1 μm. a. (inset) scanning electron micrograph (SEM) showing the densely ciliated circumoral ciliary band. Bar = 0.5 μm.

FIGURE 15. Section through a basal portion of a nerve cell giving rise to axonal processes in the ciliary band. TEM. Bar = 0.5 μm.

FIGURE 16. Transverse section through axonal tract of the circumoral ciliary band showing vessicles containing fine granular material and electron-dense material. TEM. Bar = 0.5 μm.

FIGURE 17. Longitudinal section through the axonal tract of the circumoral ciliary band. TEM. Bar = 1 μm.

length, axon tracts also course around the perimeter of the esophagus. The axons occur in groups of up to 20–25, typically underlie the epithelial cells, and do not cross the basal lamina (Figs. 22, 23).

Nerve cells outside the ciliary bands occur at the apical end of the larva in regions adjacent to the ciliary bands (Figs. 24, 25). The nerve cells are distinguished by the prominent, electron-dense Golgi body and numerous 0.05 to 0.15 μm vesicles. Axons, in loosely organized tracts underlying the epidermis, are scattered throughout this region (Fig. 26).

The cells within the blastocoel that are associated with the ciliary bands (Fig. 2) are characterized ultrastructurally by large vacuoles that contain a variety of materials (Fig. 27). In some sections these appeared to be phagosomes containing amorphous material, or whole, necrotic cells. The remainder of the cytoplasm of these cells typically contains small cisternae of rough endoplasmic reticulum, mitochondria, and lysosomes.

DISCUSSION

The principal behaviors of the auricularia are swimming and feeding (Strathmann, 1971). The ciliary bands are arranged so that a larger proportion of ciliary beat is directed posteriorly and the larva moves with its anterior end foremost. As particles pass over the ciliary band, there are localized reversals of ciliary beat which deflect the particles into the oral field. Food particles are transported to the mouth by currents produced either by portions of the lateral band or by the adoral ciliary band. There are three means by which larvae are able to reject particles: (*i*) not retaining particles

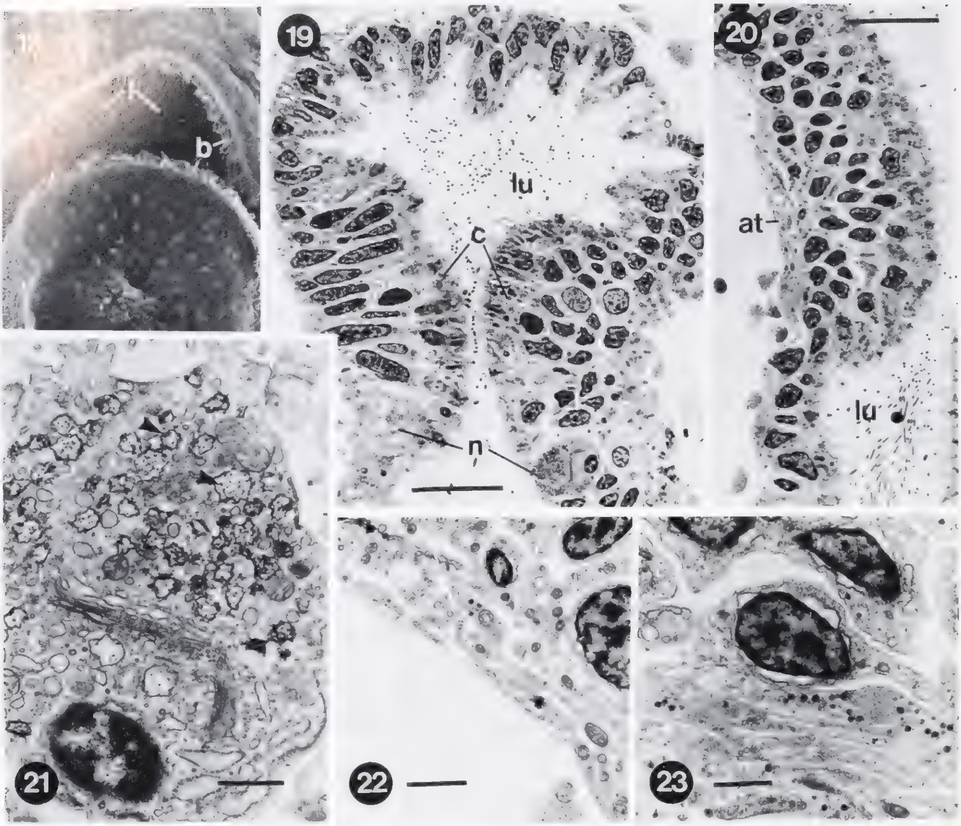


FIGURE 18. SEM of the adoral ciliary band (b) showing the palp-like thickenings (l) of the lower lip of a 23-day-old auricularia of *Parasticopus californicus.* Bar = 20 μm.

FIGURE 19. Transverse section through the lower lip showing the ciliated cells (c) and nerve cells (n) of the adoral ciliary band. lu, lumen of the esophagus. TEM. Bar = 10 μm.

FIGURE 20. Longitudinal section through the thickened region of the adoral ciliary band of the lower lip of an auricularia of *P. californicus.* at, axonal tract; lu, lumen of the esophagus. TEM. Bar = 10 μm.

FIGURE 21. Section through a nerve cell from the adoral ciliary band. Arrows indicate irregularly shaped vacuoles containing electron-dense material. TEM. Bar = 1 μm.

FIGURE 22. Section through axonal tracts at the base of the esophageal epithelium. TEM. Bar = 2 μm.

FIGURE 23. Detail of axonal tracts within the thickened epithelium of the lower lip. TEM. Bar = 1 μm.

as they pass over the circumoral ciliary band; (*ii*) overall reversal of the direction of ciliary beat, causing particles to be cleared from the oral field and the larva to be propelled backwards; and (*iii*) reverse peristalsis of the esophageal muscles causing regurgitation of the contents of the esophagus. Strathmann (1971) noted that regurgitation may be accompanied by ciliary reversal of the circumoral ciliary band as well as reversal of the adoral ciliary band. When larvae encounter an obstacle they reverse the direction of ciliary beat and back away.

Although this behavioral repertoir is not complex, it does show coordination of effectors (ciliary reversals accompanying esophageal regurgitation) and responses of effectors some distance from the point of stimulation (obstacle avoidance). These observations have led several authors to speculate that auriculariae possess a nervous

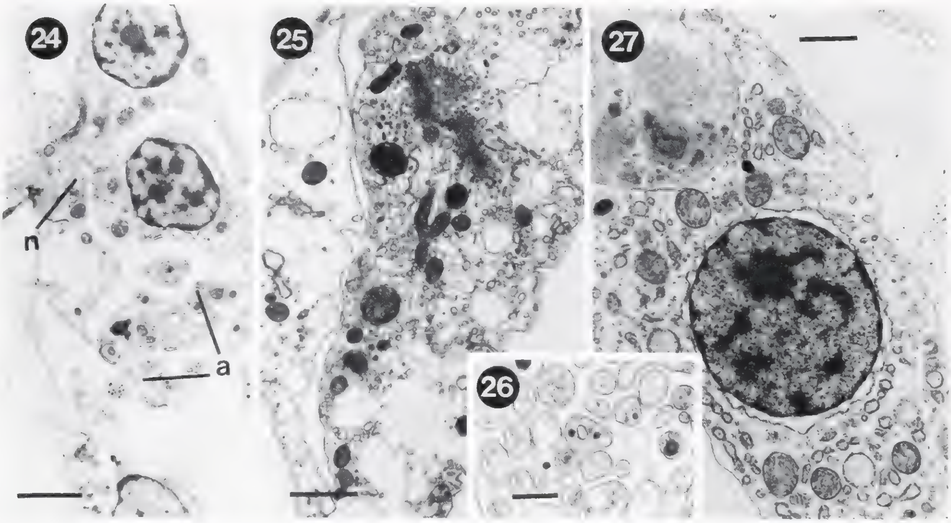


FIGURE 24. Transverse section through the epidermis at the apical end of an auricularia larva showing nerve cells (n) and axons (a) that are not within the circumoral ciliary band. TEM. Bar = 2 μm.

FIGURE 25. Detail of nerve cell from the epidermis at the apical end of a larva. TEM. Bar = 1 μm.

FIGURE 26. Detail of axons underlying the epidermis at the apical end of a larva. TEM. Bar = 0.5 μm.

FIGURE 27. Section through a blastocoelar mesenchyme cell associated with the circumoral ciliary band. TEM. Bar = 1 μm.

system (Mortensen, 1920; Garstang, 1939; Strathmann, 1971, 1975). Strathmann (1971) observed that in the presence of excess $MgCl_2$, an inhibitor of synaptic transmission in some animals, overall reversals of the ciliary band, rejection of particles from the esophagus, and localized reversal associated with feeding were blocked.

The principal effector organs of the larva are the ciliated cells of the ciliary bands and the musculature of the esophagus. The neural components observed in this study are associated with larval effectors and probably function primarily in coordinating swimming and feeding behaviors of the larva. Conceivably the nervous system mediates overall ciliary reversals and coordinates reverse peristalsis of the esophageal musculature with reversals. It is also possible that the nervous system mediates the localized ciliary reversals involved with particle capture. The sparse distribution of nerve cells within the ciliary band would argue against such a hypothesis as it would be expected that the spacing of the cells would approximate the length of band involved in a localized reversal. Although this distance is not known for sure (Strathmann, 1971), it would appear to be much shorter than the 50 to 150 μm observed between nerve cells of glyoxylic acid treated specimens.

The nervous system of the echinopluteus has been described by Ryberg (1977) and Burke (1978, 1981, 1983a, b) and is thought to consist of tracts of axons associated with the ciliary bands and esophagus along the length of which are interspersed nerve cell bodies. An apical neuropil and ganglion on the lower lip of the pluteus has also been described (Burke, 1983a). The nervous system of the bipinnaria and brachiolaria larva of asteroids includes axon tracts associated with the ciliary bands and the esophagus (Barker, 1978; Burke, 1983c). Two types of nerve cells are interspersed along the length of the ciliary band, and ganglia and neuropil are associated with the lower lip of the mouth and the brachiolarian arm. The larval nervous system of the doliolaria larva of the crinoid, *Florometra seratissima,* is described by Chia *et al.* (1986) as consisting of tracts of axons associated with the ciliary bands and an extensive apical ganglion associated with the larval adhesive organ. The neural organization of the ophiopluteus of *Ophiopholis aculeata* is similar to that of the other larval echinoderms with axonal tracts associated with the ciliary bands and an oral ganglion (Burke, unpub. obs.).

There are several similarities in the organization of these nervous systems. All the forms have axon tracts associated with the ciliary bands. In the feeding larval forms (asteroids, echinoids, and holothuroids) nerve cells are also associated with the ciliary band. In the non-feeding doliolaria the axons are associated with the ciliary bands, but no nerve cells were identified within the ciliary bands and the axons also appear to form a general plexus underlying the epidermis. All the echinoderm larvae so far examined appear to have an apical nerve center which typically takes the form of a neuropil. However in holothurians and crinoids nerve cell bodies are associated with the apical nerve center and as such it has been described as a ganglion. Only in the feeding larval form has a ganglion on the lower lip been described.

These similarities in the nervous systems of echinoderm larvae are probably homologous characteristics which underline the affinities between the larval forms. Although the larval forms of echinoderms are different in general appearance, the similarities in the manner in which they feed (Strathmann, 1971, 1975) and the organization of their ciliary bands and nervous system argues for a close relationship amongst them (Dan, 1957). In constructing a phylogeny for echinoderms, if adult characteristics and the fossil record form the basis of the phylogeny (Fell and Pawson, 1966) or if rRNA sequences are used (R. A. Raff, pers. comm.), the similarities between the echinopluteus and ophiopluteus and between the auricularia and bipinnaria must be attributed to convergence. The apparent similarity in their tissue level of organization leaves the principal difference between the forms the presence or absence of a pluteus-like larval skeleton. This suggests that the convergence is based on a relatively small number of characteristics that may have been acquired or lost independently of other larval characteristics.

Specimens prepared for immunofluorescence with anti-serotonin revealed a distinct population of nerve cells not visualized with the glyoxylic acid-induced fluorescence. Ultrastructurally these cells have several characteristics consistent with them being nerve cells, but none which distinguished them from the nerve cells revealed with glyoxylic acid. Glyoxylic acid-induced fluorescence has been suggested to be specific for dopaminergic nerves (Grace and Bunny, 1980; Sharpe and Atkinson, 1980), however, Keenan and Koopowitz (1981) have presented evidence that glyoxylic acid will induce fluorescence of L-dopa, L-dopamine, L-tyrosine, and norepinephrine with emittence spectra that are indistinguishable. In preparations of echinoplutei the pattern of nerves revealed with anti-dopamine antibodies is identical to that observed with glyoxylic acid induced fluorescence (Bisgrove, 1985). Although this does not assure specificity of glyoxylic acid-induced fluorescence, it does corroborate the interpretation that the cells are dopaminergic.

It is possible that the nerves so far identified with immunofluorescence and histochemistry do not comprise the entire nervous system of the larva. Neither technique was able to provide an image of the nerve tracts identified with TEM in the esophagus. In the echinopluteus, these nerves are immunoreactive with antibodies against gamma-aminobutyric acid (Bisgrove, 1985). The mesenchyme cells within the blastocoel associated with the ciliary band appear to be homologous with the mesenchyme cells of the echinopluteus suggested to be nervous by Ryberg (1977). There are however, no cytological characteristics that suggest they may function as nerve cells. As techniques for the identification of nervous tissues become more refined, the full extent of the larval nervous system will become clearer.

The auricularia of *P. californicus* metamorphoses to a doliolaria after about three weeks. The transformation is radical, involving an overall shrinking in the size and rearrangement of the circumoral ciliary band into four separate bands of cilia surrounding the barrel-shaped larva (reviewed by Hyman, 1955). The doliolaria is transformed gradually and directly in about a week into the pentactula which settles. It is not known how much of the larval nervous system is carried over into the adult form, though as the ciliary bands are ultimately resorbed, it is likely that at least a portion of the nervous system is destroyed as well. In other planktotrophic echinoderm larvae it appears that the nervous system of the larva is destroyed during metamorphosis (Chia and Burke, 1978). It is possible that the neural tissues are specialized for control and coordination of larval tissues and play little or no role in the development of the adult nervous system. The complexity of the nervous system of the auricularia appears greater than is necessary for control of the relatively simple larval effectors, suggesting that other roles for the nervous system may exist. Neurosecretory and neuroendocrine processes may exist which function in controlling the developmental events of metamorphosis as has been hypothesized for other larval forms (Hadfield, 1978; Chia, 1978; Burke, 1983a, d). However, other than the relatively rapid and coordinated onset of metamorphosis, at present there are no data to support such a hypothesis.

ACKNOWLEDGMENTS

This research was supported by a Natural Sciences and Engineering Research Council of Canada University Research Fellowship and Operating Grant to R.D.B. and an NSERC Post-graduate Scholarship to B.W.B. A portion of this research was undertaken at the Friday Harbor Laboratories. We are grateful for the able technical assistance provided by Brian Kingzett.

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STRUCTURE AND OPTICS OF OMMATIDIA FROM EYES OF STOMATOPOD CRUSTACEANS FROM DIFFERENT LUMINOUS HABITATS

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ABSTRACT

Shapes and sizes of ommatidia in six genera of stomatopods from different luminous habitats are described. Cornea-cone apertures and acceptance angles have been calculated. The ommatidia belong to the apposition type with fused rhabdoms as in most Malacostraca, but the spindle-shaped cone and the transparent wedges under the cornea are acquisitions of stomatopods. The same is true for rhabdom specializations, especially the thin undulated rhabdoms in ommatidia of the six-row middle band of the Gonodactyloidea, that divides the eye in two halves. Several regions can be distinguished in stomatopod eyes by differences in shapes, sizes, and proportions of their ommatidia and by the skewing pattern along the columns of ommatidia. As more light becomes available in the habitat, apertures and acceptance angles seem to decrease mainly by increasing the lengths of the cones.

INTRODUCTION

Ommatidia, their optics, visual fields, eye maps, and other parameters in insect and crustacean eyes have been described for many species (extensive surveys are given in the following articles: Kunze, 1979; Snyder, 1979; Land, 1981b; Wehner, 1981; Shaw and Stowe, 1982). The ommatidia and eyes of stomatopod crustaceans have several characteristics which differ from any other compound eye. We have started several lines of research on the structure and function of these eyes in an attempt to analyze possible integrating and range-finding mechanisms. Trying to correlate the various parameters of the single ommatidium and the ensemble of ommatidia with the luminous environments of the species, we realized that our knowledge of the luminosity in the microhabitats and of the daily activity cycles is limited. In fact, if evolutionary adaptations occur, they are not obvious and not coherent throughout the genera analyzed. In this paper we describe structure and optics of the single ommatidium of the following species:

Bathysquilla crassispinosa (Manning, 1961) (Bathsquillidea, 500+ m depth).
Echinosquilla guerini (White, 1861) (Protosquillidae, 100 m depth).
Squilla mantis (Linnaeus, 1758) and *S. empusa* Say, 1818 (Squillidae, generally less than 100 m depth).
Hemisquilla ensigera (Owen, 1832) (Hemisquillidae, *ca.* 20 m depth).
Gonodactylus spp. (several species, Gonodactylidae, shallow, tropical, coastal waters).
Pseudosquilla ciliata (Fabricius, 1787) (Pseudosquillidae, shallow, tropical, coastal waters).

Received 12 November 1985; accepted 25 March 1986.

MATERIALS AND METHODS

Dimensions of the components of ommatidia were measured from photographs [illegible] of living razor-cut eyes and from histological sections, primarily from unstained sections of eyes fixed in glutaraldehyde. A Leitz Orthoplan with or without interference optics and uv-fluorescence was used to study the sections.

We calculated the acceptance angle (which determines the visual field) according to Snyder (1979) (see below). We do not know the refractive indices of the components of the optical equipment of the ommatidia. Therefore we calculated the acceptance angle for two mean values: 1.5 and 1.4. The former value was used by Horridge and Duelli (1979). We have considered 1.4 as the lower limit because the refractive index of seawater is around 1.34. Obviously our calculations do not give precise values for the acceptance angles. But the changes determined by different dimensions of the optical system are much larger than those caused by varying refractive indices (Table II). The mean value of the refractive index of the whole optical equipment presumably would be between the two values used in our calculations. Refractive indices measured in other arthropods lie between 1.35 and 1.55. The refractive index does not vary within the cone. It does vary in the corneal layers and between cornea, the corneagenous cells, and the cone. Furthermore, the calculations following Snyder (1979) do not consider the curvatures of the corneal surfaces. It seems impossible to calculate the acceptance angle precisely considering the refraction by the cornagenous cells and the cone structure. Thus, our calculations seem to be a reasonable approximation until electrophysiological measurements are available.

The apertures, *i.e.*, the angle within which light would be admitted to the ommatidium, were calculated trigonometrically from the facet diameter and the distance between the corneal surface and the distal tip of the rhabdom. The same angle results from the contour of a longitudinal cone section. We also measured it by looking through the cornea-cone unit at an image at a distance of 3 cm and one at 1 m from the eye. The portion of the image that is transmitted through the unit indicated an aperture corresponding to the calculated aperture. Obviously, this does not mean that all light entering also will be transmitted into the rhabdom. All measurements were taken from eyes adapted to bright light. In *Squilla*, the cone widens and the rhabdom lengthens during dark adaptation (Schiff, 1974).

Comparative pseudopupil observations are described in Abbott *et al.* (1984). Because of the complex structure and optics of stomatopod ommatidia, pseudopupil measurements do not supply information about either the acceptance angle or the aperture, but only about the distribution of optical axes.

Ommatidia parallel to the middle band of specialized ommatidia are arranged in *rows*; sequences of ommatidia perpendicular to the rows are called *columns*. The parts of the cornea furthest away from the middle band are the sides and between these and the middle band are the submedian parts of the eye.

RESULTS

Stomatopods, predatory Crustacea who catch their prey with a violent strike (Caldwell and Dingle, 1976), have eyes designed presumably for monocular and binocular range finding (Schiff *et al.*, 1985). The eyes are divided into two halves by a middle band (Demoll, 1909; Schiff, 1963, 1976; Horridge, 1978) of either two or six rows of ommatidia in the species described here. Along the column the first 3–10 ommatidia on each side of the middle band are skewed towards the middle band. In this way axes of skewed ommatidia in one half of the eye cross those from the ommatidia in the opposite half. *Squilla* and *Echinosquilla* from a dim habitat have approximately cylindrical corneas divided by two rows of middle ommatidia in a groove in the former species and six rows in the latter. *Odontodactylus, Hemisquilla, Gonodactylus,* and *Pseudosquilla* from a medium to bright environment have spherical to elliptical corneas with six rows of middle ommatidia which in *Odontodactylus* and *Gonodactylus* form a crest and in *Hemisquilla* and *Pseudosquilla* a slight depression.

Although many stomatopod species live in dim to dark environments where superposition eyes would be more suitable to catching light, stomatopods possess apposition eyes. To compensate for this, the corneal facets, cones, and rhabdoms are large. Most facets are hexagonal, but square, pentagonal, and rectangular facets occur in certain regions of the eye (see below). The typical shape of a stomatopod ommatidium is shown in Figure 1. Average sizes and shapes of ommatidia from five species from different luminous habitats are drawn in Figure 2.

Four categories of ommatidia can be distinguished by the relative dimensions of the components and by their position in the eye: ommatidia from the sides, the submedian surfaces, the middle band, and skewed ommatidia on each side of the middle band.

From the middle towards the sides, ommatidia diminish in size. At the sides the cones are small or missing. These ommatidia provoke attention position (Demoll, 1909; Schiff, 1963) of eyes and striking appendages.

The shapes of ommatidia are similar in all stomatopods except for the middle ommatidia described below. Sizes and proportions instead vary within one eye in different regions. For the same regions of the eye within one species these differ according to age, *i.e.,* the size of the individual. Sizes and proportions also differ between eyes of different genera (Fig. 2 and Table I). We did not find a clear and coherent relationship between environmental light and dimensions, acceptance angles, and cone apertures. We, therefore, calculated ratios of the various parameters (Table III). But these also do not relate coherently to ambient light conditions. In the tables the species are ordered from least light available (*Echinosquilla*) to very bright (*Gonodactylus* and *Pseudosquilla*). As can be seen, neither dimensions of ommatidia, nor angles of apertures of cones and rhabdoms, nor ratios change coherently with the availability of light. The total number of ommatidia increases, possibly with the availability of light or with the size of the species, being approximately 1500 in *Echinosquilla,* 4000 in *Squilla,* 8000 in *Hemisquilla,* 10,000 in *Odontodactylus,* but only about 5500 in *Gonodactylus.*

Ommatidia in the submedian eye parts

Corneal facets. Corneal facets are hexagonal in all regions of the cornea except for the middle bands and adjacent rows of ommatidia.

The cornea in all stomatopods is composed of many refracting layers (each 1.7 μm thick in *Odontodactylus*). In *Odontodactylus* and *Hemisquilla* strongly refracting double layers have been observed (Fig. 3) at the inner as well as the outer surface of the corneal facet. The distance between the peripheral double layer and the main part of the cornea is 4.3 μm in the center of the facet and 2.3 μm at the transition zone with the neighboring facet. The separation within the double layer is 0.3 μm.

The outer surface curvature of the facets varies within each eye and between genera. Species from a bright environment, such as species of *Odontodactylus, Gonodactylus,* and *Pseudosquilla* have relatively flat outer surfaces of the corneal facets, while *Echinosquilla, Squilla,* and *Hemisquilla* (dark, dim, and medium light habitats) have strongly convex outer facet surfaces. A measure of convexity is given in Figure 2 and Table I showing the thickness of the corneal facet (distal to proximal surface) in the

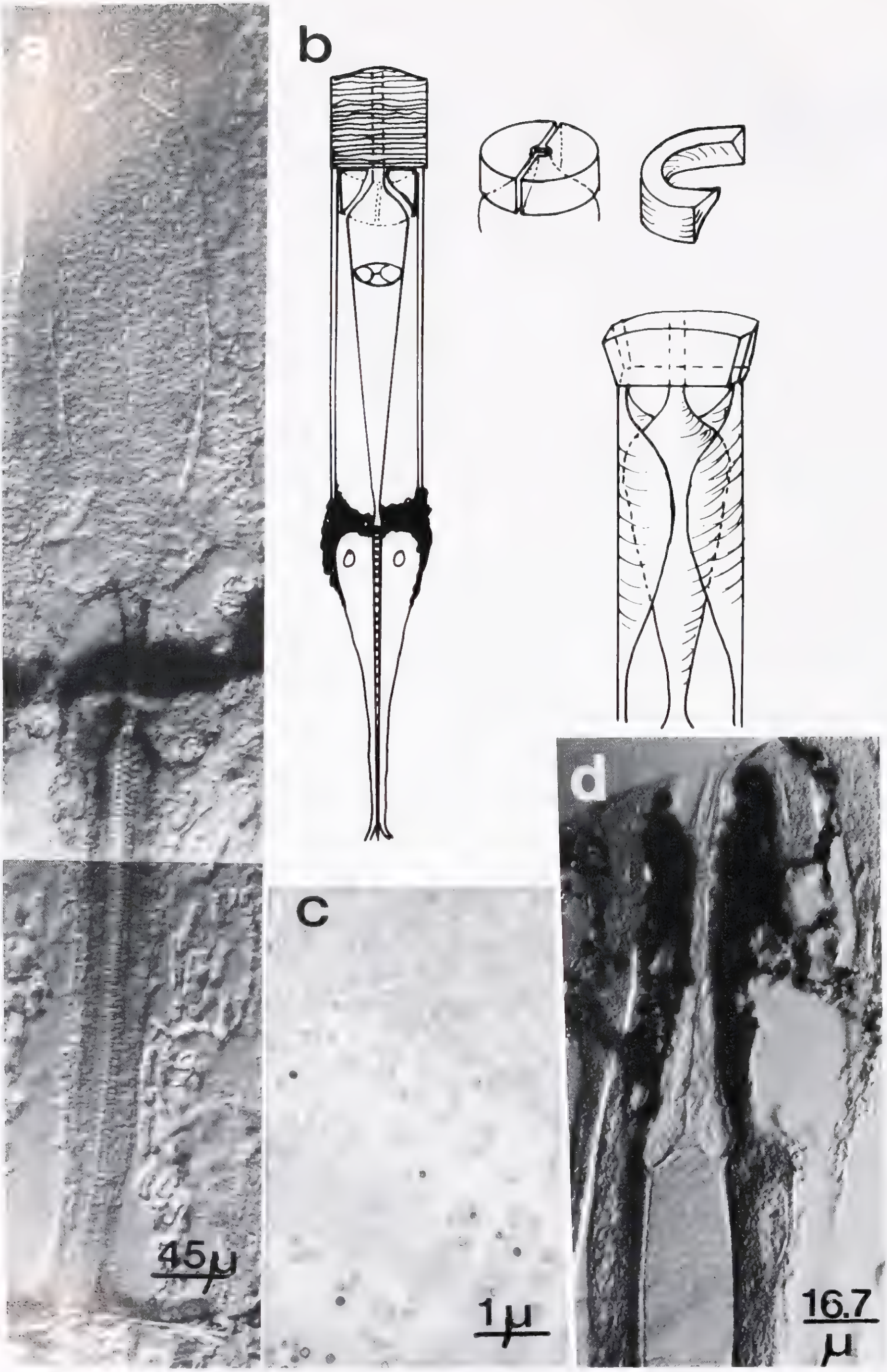
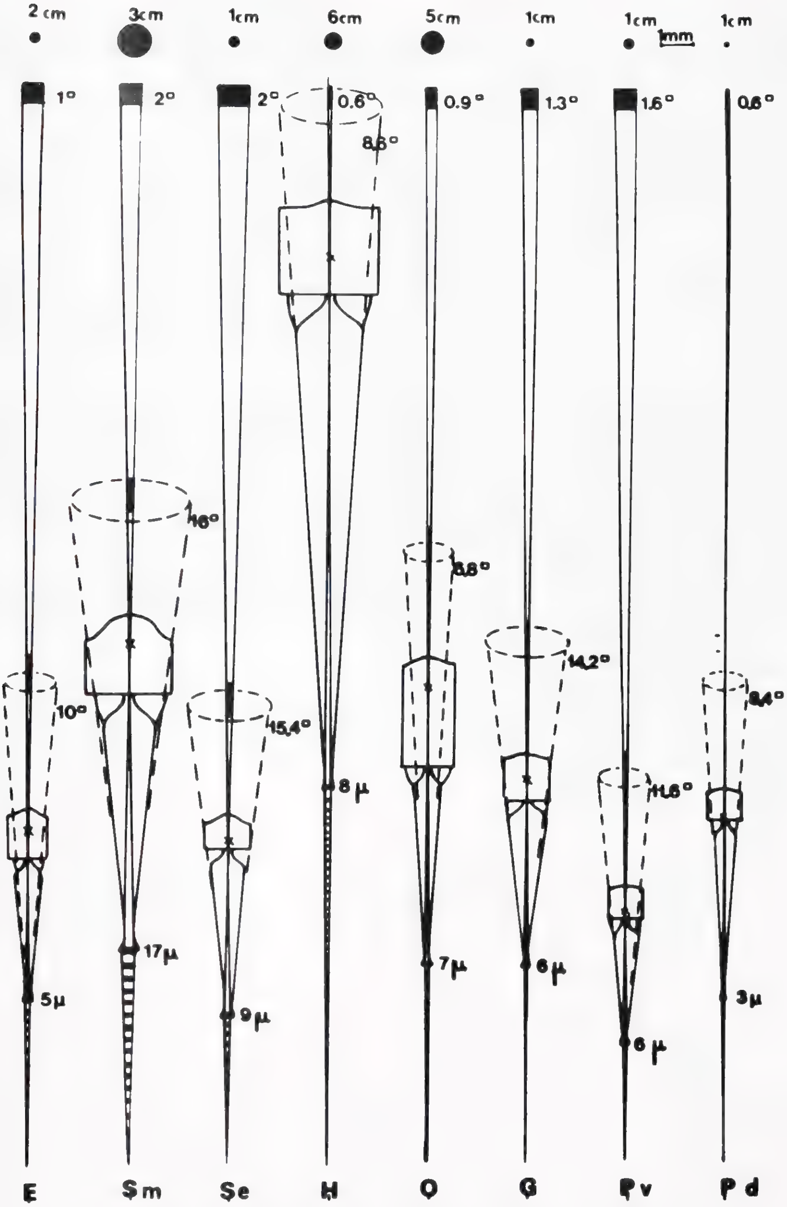


FIGURE 1. The stomatopod ommatidium: (a) an ommatidium of *Pseudosquilla ciliata,* unstained section; (b) diagram of an ommatidium of *Odontodactylus scyllarus* and on the right: the transparent wedges formed by the corneagenous cells and the transparent membranes formed by the accessory pigment cells surrounding the cone; (c) electron microscope photograph tangential to the rhabdom surface from the eye of *Squilla mantis.* The lower part of the photo shows the regular pattern of bridges and columns stretching between the thin layer on the rhabdom surfaces and the main bulk of the retinular cells. (d) Cone-rhabdom junction in an ommatidium of *Squilla mantis* (unstained). Bars and numbers indicate micrometers.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Pv
Pd

FIGURE 2. Ommatidia of: E: *Echinosquilla guerini;* Sm: *Squilla mantis;* Se: *Squilla empusa;* H: *Hemisquilla ensigera;* O: *Odontodactylus scyllarus;* G: *Gonodactylus,* posterior ommatidium. The frontal ommatidia are similar in dimensions to the frontal ones in *Pseudosquilla* Pd. Pv: posterior and Pd: anterior ommatidia of *Pseudosquilla.* Indicated in micrometers are the diameters of the distal rhabdoms; in degrees on top: acceptance angles, in the middle (dashed lines) the cornea-cone apertures. Above the ommatidia the visual fields are shown at the indicated distances from the cornea together with the scale for the visual fields. Distances chosen are those at the approximate striking distances of the species.

TABLE I

Dimensions of components of ommatidia in micrometers

	Facet diameter			Corneal thickness		Cone maximum diameter			Cone length			Rhabdom maximum diameter			Rhabdom length		
	mi		si	mid.	mar.	mi		si	mi		si	mi		si	mi		si
Echinosquilla		57		73	60		36			208			4.5 ± 0.2			246	
Squilla mantis		128		125	75		77			390			17.8 ± 2.3			323	
Squilla empusa		66 ± 2.7		46 ± 2	38 ± 2		47 ± 3			246 ± 20			9.4 ± 0.3			204 ± 3	
Hemisquilla		110		150	145		100			745		11 ± 0.9	6.8 ± 1.4*	8 ± 2	607 ± 50		570 ± 13
Odontodactylus		115		165		63 ± 18	24*	36	445 ± 89		305 ± 6.6	11 ± 3		6.8 ± 0.2	473 ± 78		302 ± 10
Pseudosquilla																	
front		40		40	39		19*	31		270			2.5*	3.1			194
dorsal middle		50		50			40			176 ± 13		11.6 ± 2.1		5.6 ± 1.6	233 ± 14		179 ± 54
Gonodactylus																	
front	40		35	60		30	14*	18		230			3			630	
dorsal middle		70		68	65		58.5 ± 6.6		289 ± 13	310*	242	6.3 ± 2.9		6.9 ± 0.4	293 ± 45		295

Hemisquilla, undulated rhabdom: distal diameter = 7.5 μm; main part, diameter = 6 μm; proximal diameter = 1–1.5 μm.
± = value of standard deviation σ.
mi = data for the ommatidia in the middle band.
si = data for the ommatidia to the sides of the middle band, *i.e.,* ommatidia in the submedian parts of the eye.
* = data for the skewed ommatidia adjacent to the middle band.
mid. = middle of corneal facet.
mar. = margins of corneal facet.

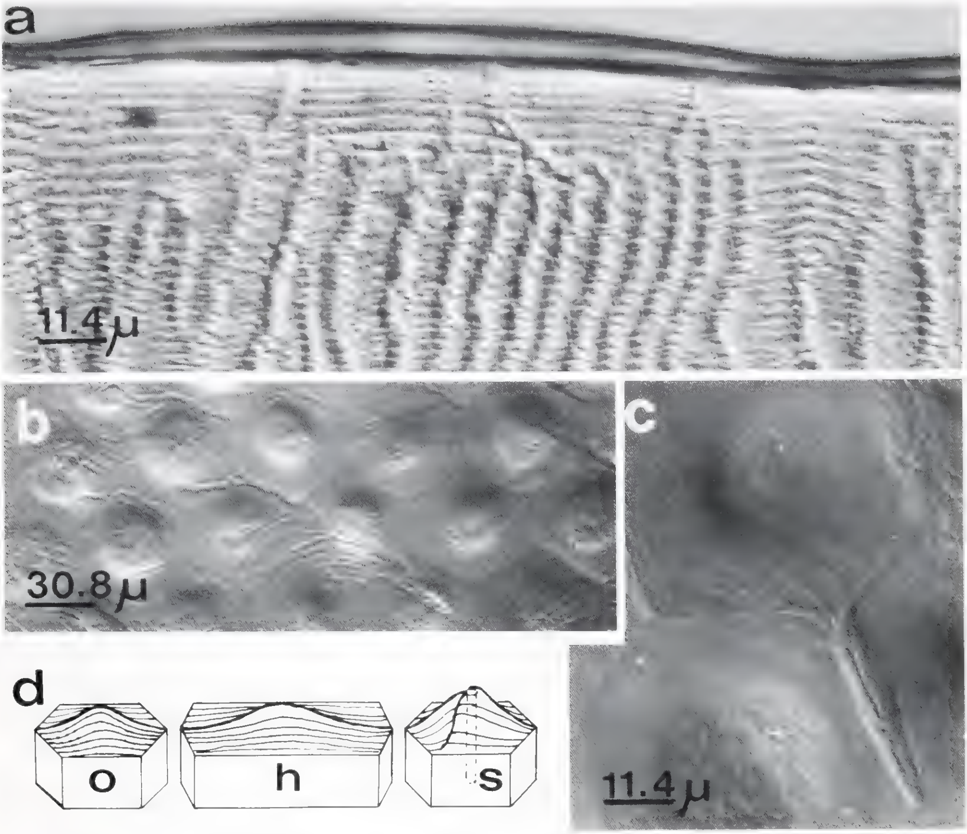


FIGURE 3. The facet surfaces: (a) part of a section through a facet of the *Odontodactylus* eye. Layered structure can be seen and the proximal and distal double layers with different refractive indices (Nomarski optics). (b and c) Tangential sections through facets of the eye of *Pseudosquilla ciliata;* (d) diagrammatic drawings of facets of (o) *Odontodactylus;* (h) *Hemisquilla;* (s) *Squilla.* All sections unstained in polarized light.

center of the facet compared to that at the margins in the different genera. Surface convexity usually is not homogeneous over the facet surface, but a facet is flat in the periphery and rises rather abruptly towards the center (Fig. 3). The surfaces are usually complicated. For example, the facets in *Squilla* show ridges and a small depression in their center. *Hemisquilla* has double ridges, zig-zagging along the rows near the rather flat margins of the facets. In *Gonodactylus* each row of the ommatidia of the middle band has a different shape, and the facet surfaces have different curvatures as well (see also Manning *et al.,* 1984, and, for an extreme case of curvature, Schiff and Manning, 1984).

Cones. Cones in stomatopod eyes have a shape not described for other arthropods. At its distal end the cone slims down abruptly to a 10 μm wide tube, 15 to 20 μm long (*Squilla mantis*) which attaches to the middle of the corneal facet. The space left between the cornea and the widest part of the cone is occupied by the corneagenous cells which form two semicircular wedges around the distal cone extension (Fig. 1). Interference microscopy and observation of the living eye indicate different refractive indices for the corneagenous cells and the cones. The membranes of cones and corneagenous cells seem to have indices different from those of the intracellular contents.

The cones are built up of four parts delimited by membranes. At its proximal part the cone slims down to a diameter of about 10 μm but then, unlike other arthropod ommatidia, widens again and fits with a concave tip over the convex distal tip of the rhabdom. Four extensions of the cone run down along the rhabdom (Schiff and Gervasio, 1969; Schönenberger, 1977). The maximal diameter of the cones and their lengths are given in Table I, the calculated cone apertures in Table II. The acceptance angle determines the visual field of the ommatidium. The cornea-cone aperture is much larger than the acceptance angle and indicates the solid angle within which light will enter the ommatidium. Part of this light will be reflected or absorbed by pigments at the retinal surface. Light from the small, central visual field will reach the rhabdom. A large aperture means that also oblique rays from the visual field will be funnelled into the rhabdom. It is obvious from Table II that no coherent relationship exists between the cone aperture and ambient light conditions.

The corneal lenslet transmits an image of a wide field corresponding to the cornea-cone aperture (Table II). The image is distorted by spherical aberration. The aberration is corrected in the cones (plus corneagenous cells) which transmit an inverse, side-reversed image with a width corresponding to the cone aperture. The cone focuses light at the level of the rhabdom and little light gets out of the sides of the cone.

In *Gonodactylus* and *Pseudosquilla* the largest surface of the cornea is usually kept dorsally, parallel to the ground and aligned with the body axis. Cone and rhabdom sizes and the relative apertures and acceptance angles are quite different for the front and back halves of the cornea. Whereas the backward and upward looking ommatidia in the proximal part of the eye are similar in dimensions and fields to those of dim light species, the forward looking ommatidia in the front part of the eye are long and slim with small visual fields and apertures (Table II).

In the eyes of some species of *Gonodactylus* and in *Pseudosquilla ciliata* the cone layer as a whole is rather transparent so that one can see the whole retinal surface from outside. Light in these cases gets through the cone walls and is then reflected and absorbed by the pigments on and in the distal retina. In other stomatopods the retinal surface is not seen as a whole because most of the light remains within the cones.

The rhabdom. The rhabdom is a typical fused rhabdom as in most Malacostraca. The number and thickness of layers of perpendicular microvilli differs between species: *Squilla mantis* has only 150 layers each 2 μm thick, but *Hemisquilla ensigera* has 1100 to 1200 layers 0.3 μm to 0.5 μm thick. Using electron microscopy, a thin layer of smaller microvilli in *S. mantis* was observed just under the cone tip which continues for the first few layers along the sides of the rhabdom. The small microvilli have a diameter of only half that of the normal (1000 Å) microvilli.

Retinular cells. Seven retinular cells surround the rhabdom and exit through the basement membrane. Here they lose their pigment (brown in the living eye) and become first order nerve fibers. In *Gonodactylus* these last are still pigmented for some 30–40 μm. Around the retinular cells are the accessory pigment cells (black). The retinal surface in all stomatopod eyes is covered with white, oily droplets. The white pigment is also contained in vesicles in the distal tips of the accessory pigment cells. The vesicles have openings towards the retina surface through which the pigment exits. A green, glittering, crystalline, strongly light-reflecting pigment lies on the retinal surface evenly distributed over the whole surface. In *Hemisquilla* the green and white pigments are concentrated only near the lateral margins with the stalk. The fifth pigment common

TABLE II

Acceptance angles and cornea-cone apertures

	Micron focal length f n = 1.5 (n = 1.4)			*Degrees* acceptance angles n = 1.5 (n = 1.4)			*mm* diameter of vis. field of 3 cm distance			of:	*cm* diam. vs. field at distance			*cm* vis. field of diam. 0.3 mm at distance of			*Degrees* cornea-cone aperture		
	mi		si	mi		si	mi		si		mi		si	mi		si	mi		si
Echinosquilla		251 (269)			1.03 (0.96)			0.5		2		0.3			1.9			10	
Squilla mantis		461 (493)			2.2 (2.07)			1.0		3		1.0			0.8			16	
Squilla empusa		261 (279)			2.08 (1.93)			0.98		1		0.3			0.9			15.4	
Hemisquilla		799 (857)		0.8 (0.74	0.5* 0.46*	0.6 0.54)	0.4	0.2*	0.3	6	0.8	0.46*	0.54	2.4	3.9*	3.3		8.6	
Odontodactylus	544 (584)		420 (450)	1.2 (1.08)		0.9 (0.87)	0.6		0.4	5	0.9		0.7	1.6		2.1	10.4	2.4*	6.8
Pseudosquilla																			
front	297		277		0.52* (0.48)	0.64 (0.57)		0.24*	0.3	1		0.08*	0.1		3.7*	3			8.4
dorsal middle	253 (216)		197	2.6 (3.08)		1.6 (1.49)	1.2		0.8	1	0.4		0.3	0.7		1.2	12.6		11.6
Gonodactylus																			
front		311		0.57	0.37*	0.5		0.26		1		0.17			1.7			10	
dorsal middle	328 (342)		280 (297)	1.1 (1.06)		1.3 (1.2)	0.5		0.6	1	0.2		0.2	1.7		1.5	12		14.2

mi, si, * as in Table I.

TABLE III

[illegible] *of components of ommatidia*

	Length of cone length of rhabdom			*Length of cornea + cone* facet diameter			*Rhabdom length* rhabdom diameter			*Cornea-cone aperture* acceptance angle		
	mi		si	mi		si	mi		si	mi		si
Echinosquilla		0.9			4			54.7			10	
Squilla mantis		1.2			3.6			18			7	
Squilla empusa		1.0			3.7			21.7			7	
Hemisquilla	1.2		1.3		6.6		55	83*	71	11	17*	14
Odontodactylus	0.9		1.0	4.7		4.7	43		44	9		8
Pseudosquilla												
front		1.4			8			67			13	
dorsal middle	0.8		1.0		4.4		20		32	5		7
Gonodactylus												
front		0.4		17		20		210			20	
dorsal middle	1.0		0.8		4.5		47		48	11		11

mi, si, * as in Table I.

to all stomatopods is bright red and crystalline. The crystals are contained in threadlike structures (Schiff and Gervasio, 1969; Schönenberger, 1977).

A thin (2.3–0.3 μm) layer of retinular cells adheres to the rhabdom. This layer is connected to the main bulk of the cells by cytoplasmic bridges and columns (0.2 μm wide, 0.6 μm long) all enveloped by the cell membrane and distributed in a very regular pattern (Fig. 1) (for *S. mantis* see Schiff and Gervasio, 1969). Therefore there is an apparently empty space between the rhabdom and the bulk of the retinular cells. Similar columns have been found in other Crustacea (Shaw and Stowe, 1982) and seem to be present in all stomatopod ommatidia (except in the 'cube-thread' rhabdom, see below).

Ommatidia in the middle band and skewed ommatidia adjacent to the middle band have different structures and we will describe them in a separate chapter.

Ommatidia in the central part of the eye

All the ommatidia in the *Squilla* eye are similar in shape but those in the two rows of the middle groove are slightly larger. In contrast, the ommatidia in the six rows of the middle band of the spherical and the elliptical eye of other genera are distinctly different from all the other ommatidia. Also the skewed ommatidia in the first four to five rows on each side of the middle band show differences with respect to other ommatidia, mainly because they are longer and slimmer.

The six-row middle band (Fig. 4)

Cornea. In *Gonodactylus* the corneal facets in the middle band have different surface curvatures for each row, and thus bulge out to different degrees. *Hemisquilla, Gonodactylus, Pseudosquilla,* and, especially *Odontodactylus,* have rectangular surface facets with their longer dimensions across the middle band (*Odontodactylus:* 150 μm across, 80 μm along the middle band). In *Gonodactylus* facets also differ in size in the different rows. There are two rows of ommatidia with large facets (30 μm × 14 μm), one with larger (33 μm × 18 μm), one with smaller (24 μm × 15 μm) and finally one with larger (30 μm × 18 μm) facets. The corneal facets of the first 3 to 5 rows of

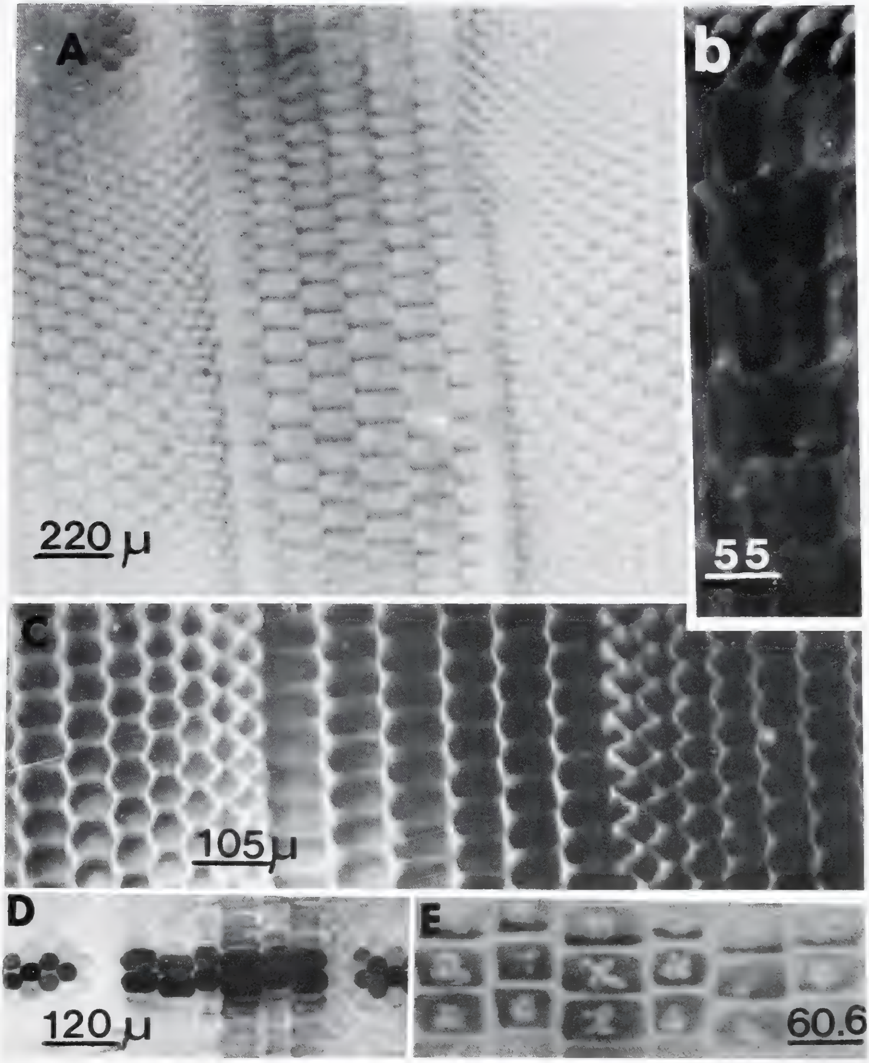


FIGURE 4. Facets in the middle band: (a and b): in *Odontodactylus;* (c): in *Hemisquilla* (focusing slightly below the surface); (d and e): in *Gonodactylus.* In (e) notice the light patterns in the depth of the pseudopupil. These patterns can be seen in most stomatopod pseudopupils, but have different characteristic shapes and sizes in different species. In *Gonodactylus* they are particularly large and cloverleaf shaped.

ommatidia adjacent to the middle band are small (Table I) and square, then gradually grow and become pentagonal and finally hexagonal as are all the remaining facets. The small facets of the three to six rows adjacent to the middle band belong to skewed ommatidia with optical axes convergent between the two halves of the eye.

Cones have the same shape, though not the same dimensions, everywhere.

The rhabdoms and retinular cells. The rhabdoms of five of the six rows of middle ommatidia are considerably larger than in the other ommatidia and also differ in shape. Across the middle band the first two rows of ommatidia have rhabdoms with

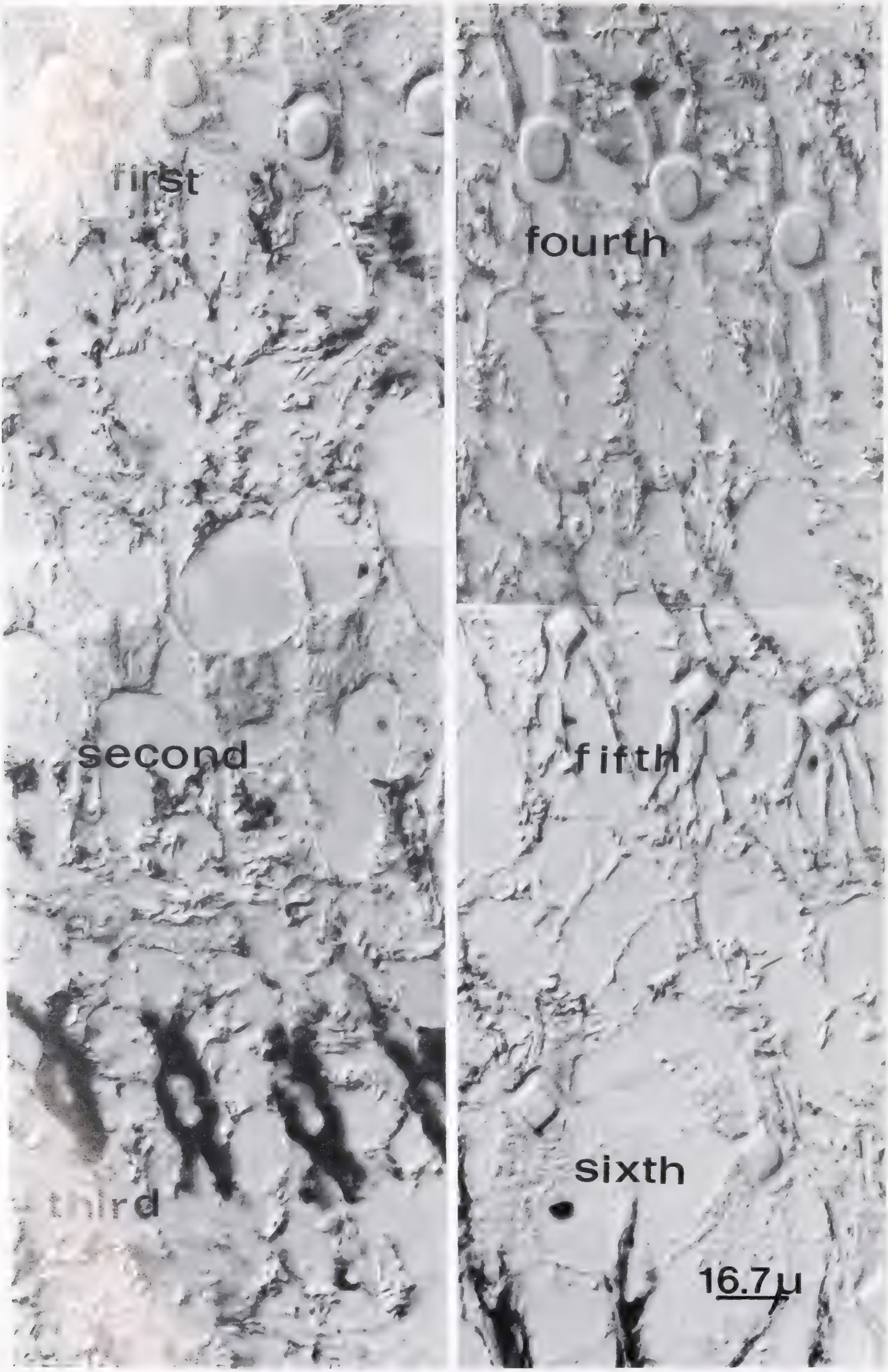

first
second
third
fourth
fifth
sixth
16.7µ

FIGURE 5. Cross section (unstained) through the rhabdoms in the middle band of the eye of *Odontodactylus*. The darkly pigmented ommatidia (third row from the top) are the "cube-thread" ommatidia.

square sections (but larger than elsewhere in the eye). This is followed by one row with round cross sections, one row with a 'cube-thread' (see below) rhabdom, one row with round cross sections and finally one with square cross sections (Fig. 5).

The retinular cells in ommatidia in the middle band are larger than those in the remaining part of the eye. The amount of pigment in the retinular cells is small and different for each row in the middle band. It is concentrated mainly around the rhabdoms in the light-adapted state. The pigmentation varies also with species and genus.

In one row next to the 'cube-thread' ommatidia in the eyes of *Gonodactylus* and *Pseudosquilla*, a small section between cone and rhabdom shows up bright red (light filter?) in unstained, glutaraldehyde-fixed preparations.

The 'cube-thread' rhabdom. A rhabdom has been observed in *Hemisquilla* and *Odontodactylus* which we have called the 'cube-thread' rhabdom (Figs. 6, 7), referring to its appearance in sections: 3 μm wide cubes at regular distances, connected by a thin (0.1 μm) thread. A reconstruction from serial sections shows it to be an undulated rhabdom which in a section appears as a thread beaded with little cubes at regular distances. The cubes are cross sections through the 3 to 6 μm wide rhabdom, while the threads are a small unpigmented space between two neighboring retinular cells. As the retinular cells are heavily pigmented, the spaces between neighboring cells show up lighter. We think that the undulation of the rhabdom, and only of the rhabdom but not the retinular cells, is not an artifact for the following reasons: if the zig-zagging were an artifact caused by fixation, a living and straight rhabdom would be much longer than the available space between cone and basement membrane. Furthermore, the spacing is very regular and a membrane envelopes the rhabdom. At the distal and proximal ends of the rhabdom the undulation can be observed directly (Figs. 6, 7) and the retinular cells are clearly not undulated.

The 'cube-thread' rhabdom seems to be present also in *Gonodactylus* and *Pseudosquilla* and may exist in all eyes with six rows in the middle band.

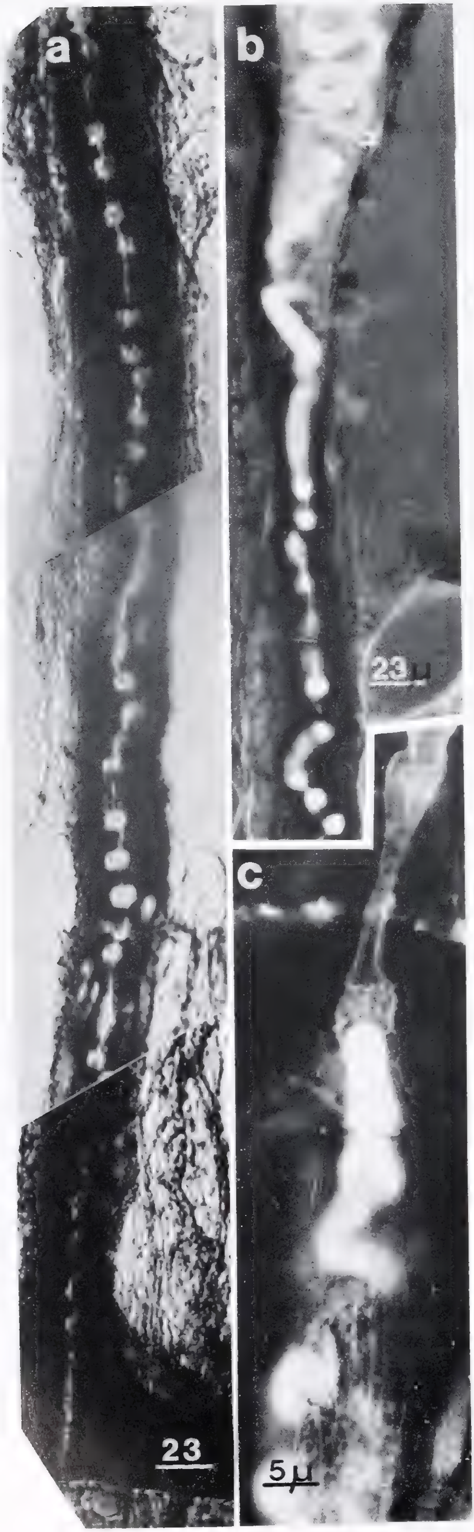
At the distal end of the ommatidium the retinular cells do not surround the rhabdom up to its junction with the cone but turn away from it more proximally. Therefore, a large part of the distal rhabdom is not covered by pigmented cells and can be easily observed. It is tightly waved (Fig. 6) and enveloped by a membrane which starts at the cone junction. The distal rhabdom is larger and clearly layered (Figs. 6, 7).

Though the proximal rhabdom is surrounded by the retinular cells, it is observed readily because, being thinner and undulated less tightly, all of it fits into a single section (Figs. 6, 7). While the retinular cells are straight, the rhabdom is undulated down to the basement membrane.

Proximal to the basement membrane the axons form small bundles, each bundle running in a different direction. Also, the sub-basement bundling pattern is different from that in the other ommatidia (described for *Squilla mantis* in Schiff *et al.*, 1986a). As shown in Figure 7, each axon from one side of the rhabdom, joins two from the other sides of the rhabdom. The seventh axon from the fourth side of the rhabdom probably also joins one of the bundles, but cannot be seen in the same section. Each bundle then runs in a different direction towards the lamina. While the retinular cells become thin when passing through the basement membrane, they bulge out like a cell proximal to it (Fig. 7). After this significant enlargement they become normal nerve fibers again and gradually loose their pigmentation (Fig. 7).

Visual fields and optical axes of the ommatidia

Table II gives the acceptance angles of the rhabdoms (half width of the sensitivity curve) calculated following Horridge and Duelli (1979) and described in more detail


a
b
c
23
23μ
5μ

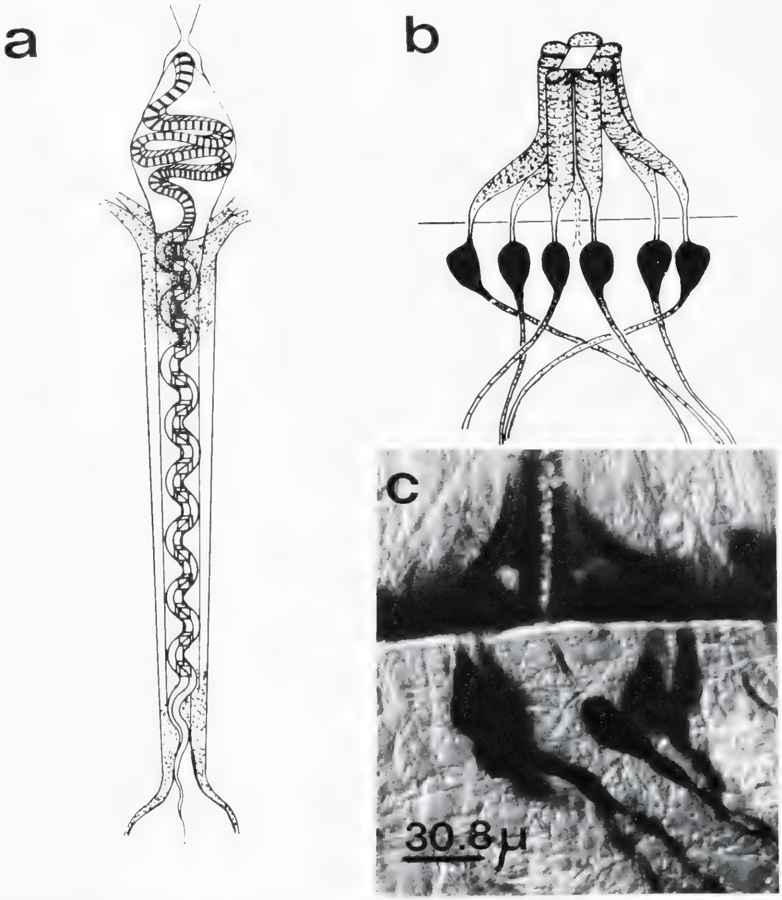


FIGURE 7. (a) Diagram reconstructed from serial sections of the "cube-thread" rhabdom. The little cubes indicate what is seen in a section like Figure 7a. (b and c) Show the proximal part of the c-t ommatidium with the thin fibers passing through the basement membrane, the cell-like bulging of fibers and the subsequent grouping of first order fibers (unstained section).

in Schiff *et al.*, 1986a). The acceptance angle or half width of the sensitivity curve is given by:

$$(\Delta\rho)^2 = \left(\frac{\lambda}{D}\right)^2 + \left(\frac{d}{f}\right)^2$$

where $\frac{\lambda}{D}$ represents the diffraction component (λ = wavelength of light, D = diameter of the corneal facet). In stomatopods the facets are so large that the $\frac{\lambda}{D}$ component can

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FIGURE 6. (a) The "cube-thread" rhabdom (unstained); (b) the distal, layered part of the c-t rhabdom surrounded by a membrane; (c) the cone juntion with the c-t rhabdom. In the lower part of the figure the rhabdom-surrounding membrane is in focus. (b and c) Photographed in ultraviolet light. The rhabdoms and cones are fluorescent in UV light especially in preparations fixed in glutaraldehyde.

be neglected and the acceptance angle calculated from the anatomical component $\frac{d}{f}$, where d is the diameter of the distal rhabdom surface and f is the value of the lens system calculated from:

$$f = \frac{n_0}{n_1} \text{ times (distance from corneal surface to distal rhabdom)}$$

(For the theoretical treatment of the optics in arthropod eyes see Snyder, 1979.)

n_0–refractive index of seawater taken as 1.34.
n_1–refractive index of cornea and cone. As we do not yet know the refractive indices of the components of the optical system the acceptance angles were calculated for mean refractive indices of 1.5 and 1.4 (in brackets) in Table II.

$\frac{d}{f}$ = acceptance angle in radians.

Also indicated in Table II are the diameters of the visual fields at a distance of 3 cm and at the estimated striking distance of the species. These values are compared for the different genera in Figure 2, where the cornea-cone apertures also are indicated. With the exception of *Echinosquilla* and considering only the front part of the eyes in *Pseudosquilla* and *Gonodactylus,* we find that the acceptance angles diminish as more light becomes available to the species. No large differences between species of the same genus have been found.

Because the functioning of a compound eye is determined by the properties and orientation of the individual ommatidia as well as by the ensemble of ommatidia and by their neural connectivity, we have studied the patterns of skewing of optical axes and part of the neuroanatomy. Skewing of ommatidia was studied both by pseudopupil observations and by histological measurements. No significant deviation occurs between optical and anatomical axes (Schiff *et al.*, 1985). The neuroanatomy of *Squilla mantis* has been described (Hanström, 1924; Schiff, 1976; Strausfeld and Nässel, 1981; Schiff *et al.*, 1986a) and from preliminary results it seems that it is not much different from the other stomatopods.

Along the columns of the eyes of all stomatopods are three long sections—one in each hemisphere of the eye and one in the middle band—in which optical axes are nearly parallel to each other, *i.e.*, with zero interommatidial angles, giving rise to the typical stomatopod triple pseudopupil (Abbott *et al.*, 1984). Optical axes of these ommatidia are perpendicular to the surface of the middle band and visual fields overlap strongly. The ommatidia separating the three sections from each other are skewed, to a different degree for each row, towards the middle band (Schiff *et al.*, 1985, 1986b; Schiff and Candone, 1986).

Along the rows, angles between two and five degrees separate the visual fields of the columns from each other in most stomatopod eyes. Smaller acceptance angles usually are associated with smaller interommatidial angles (*Hemisquilla*) and larger acceptance angles with larger interommatidial angles (*Squilla*).

Few large fibers (Tan 2 in Strausfeld and Nässel, 1981; T 3 in our description: Schiff *et al.*, 1986a) run vertically over the lamina, sending horizontal branches between the rows of cartridges, thus forming a horizontal-vertical matrix mirroring the columns and rows of ommatidia. The horizontal branches contact the cartridges along their path.

DISCUSSION

The different and quite complicated shapes of the facet surfaces, the shapes of the cones, the corneageous cell wedges, and the specializations at the cone-rhabdom junctions make it practically impossible to treat the optics theoretically. However, we know (at least for *Squilla mantis*) from direct observation that an image is transmitted which can be seen throughout the cone, varying only in size. Furthermore, we know that the image is distorted (curved) at the margins of the facet after passing through the corneal lenslet alone. This is corrected after the passage through the wedges of corneagenous cells. These permit the marginal rays to enter the cone. This means that the complex surfaces of the facets, the different refractive indices, and shapes of the components of the optic ensemble constitute an efficient lens system forming a clear image at the rhabdom. Land (1981a) measured refractive indices in cones of deep sea amphipods, and found that their refractive indices change in different parts of the cones. However, in stomatopods the rays may be funnelled to the rhabdom by the wedges without the need for the differential refraction within the cone: different indices in the cornea, wedges, and cones could explain the image formation, and, judging from interference microscopy, indices are, in fact, different.

If we calculate the visual fields of ommatidia of stomatopods we find that they are quite small, the large facets being compensated by long cones. We also find that the solid angle defined by the acceptance angle matches the proximal and distal cone extensions. Thus the paraxial rays would not pass through the wedges but enter the cones directly from the corneal facet. More oblique rays, coming from within the visual field, would pass through the wedges to be focused at the level of the rhabdom. This arrangement would have the advantage of using light within the large solid angle of the cornea-cone aperture (important for animals from dim environments), still preserving a small visual field (important for resolution of moving objects).

The wide facets with large apertures and wide rhabdoms allow a large amount of light to reach and enter the rhabdom. If the rhabdom is long, light passes through more membranes with photopigment than in shorter rhabdoms. Therefore, more light can be absorbed in a long rhabdom. Land (1981a) has shown that the number of photons per second s_a the rhabdom will receive and can absorb from an extended source emitting $L \cdot m^{-2} \cdot s^{-1} \cdot sr^{-1}$ photons is:

$$s_a = L\left(\frac{\pi}{4}\right)^2 \cdot \left(\frac{D}{f}\right)^2 \cdot d^2 \cdot (1 - e^{-kx}) \text{ or approximately:}$$

$= L\, D^2(\Delta\rho)^2$ for long rhabdoms with negligible diffraction, with D = lens diameter, d = rhabdom diameter, f = focal length, k = absorption coefficient, $\Delta\rho = \frac{d}{f}$ = acceptance angle, and x = length of rhabdom. Thus the efficiency of absorption of photons in the rhabdom increases with the diameters of corneal lens and rhabdom and with the length of the rhabdom.

A more efficient use of available light is also achieved by optical and neural pooling. Many visual fields in each column overlap (optical pooling) and the T 3 fiber, found also in *Pandalus* (Nässel, 1975) and amphipods (Land, 1981a), may be responsible for neural pooling, as suggested also by Land (1981b).

In *Gonodactylus* and *Pseudosquilla* ommatidia in the front and back of the eye differ in their optical properties. While proximal ommatidia have wide acceptance angles and short cones, the distal (front) ommatidia have small acceptance angles, long cones, small apertures, and long thin rhabdoms. It would seem, therefore, that the ommatidia in the back are better suited for a working range in low light intensities and the front ommatidia for bright light ranges. The ommatidia in the back of the eye are looking towards the crevices where the animals hide. The ommatidia in the front are looking forward into the bright light.

From these data it would seem that stomatopods, though sharing apposition eyes with animals from bright habitats, during their evolution may have adapted to dim or dark environments (wide apertures). Those species which successively occupied brighter habitats had to redevelop adaptations to avoid excessive light and saturation of receptors. These adaptations may be filters between the cone and rhabdom (red stoppers in *Gonodactylus*), changing of the proportions (for example increasing the lengths of the cones) and other, more subtle features (for example different refractive indices as in *Pseudosquilla* which might permit light to exit from the cones before reaching the rhabdom).

The meaning and importance of the middle band and its ommatidia still escapes an interpretation. The larger rhabdoms and facets may, in some cases, constitute a foveal region (Horridge, 1978), but for most ommatidia the calculated acceptance angles are not smaller and the interommatidial angles are zero. This is similar to the situation in amphipod eyes and, according to Land (1981a), increases the ability to detect small objects and/or low contrast against a homogenous background. Neuroanatomical and behavioral clues indicate that the middle bands are involved in binocular range-finding for longer distances than the monocular mechanism (Schiff *et al.*, 1985, 1986a, b).

We did not find any discussions in the literature about the optical implications of the different shapes of rhabdoms (for example square against round cross sections). Twisted rhabdoms have been described (Smola and Wunderer, 1981) and treated theoretically (McIntyre and Snyder, 1978), but the cube-thread rhabdom is not twisted but undulated. The cube-thread rhabdoms may constitute reference points for moving targets or adapt the whole eye to the environmental light. Having a very long rhabdom it could absorb more light before getting saturated, or in other words: it would have a wide range between minimum and maximum absorption.

The linear organization of ommatidia which produces the long, thin, triple pseudopupil (Abbott *et al.*, 1984) is repeated in each neighboring column. These columns are separated by interommatidial angles that are greater than the calculated acceptance angles of the individual ommatidia (Table II). It is possible that the enhanced sensitivity of the pooled columnar input coexists with a high resolution in the horizontal direction by suitable inter-columnar neural differentiation. The combination of wide facets with small acceptance angles, as well as the combination of pooled information along the columns *versus* high resolution along the rows, may work together to provide high sensitivity with little loss of resolution.

We hypothesize that the double eye, overlapping fields, and skewing patterns of ommatidia constitute a monocular range-finding device (Schiff *et al.*, 1985). Inputs are integrated in the T 3 fiber and transmitted as the visual cue for the strike used to catch prey. As vertically, along the columns, high sensitivity is achieved by the overlapping fields, an integrative mechanism for range-finding could function also in dim light. But, as the composite fields of columns are separated by inter-ommatidial angles greater than the acceptance angles along the rows, resolution in the horizontal direction may result between the global, integrative range-finders of each column.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of this research by Consiglio Nazionale delle Ricerche/National Science Foundation grants 77.01544.63, 78.02741.63, and 80.02404.02, and NATO grant 1363 to H. Schiff. H.S. also acknowledges support from the Office of Fellowships and Grants, Smithsonian Institution, during her visit in February 1983. The Institute of Anthropology, University of Torino, provided working space and the use of a microscope, and Mr. L. Burzio of that institute printed the photographs.

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THE CLOSER MUSCLE IN THE DIMORPHIC CLAWS OF MALE FIDDLER CRABS

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ABSTRACT

The paired claws in male fiddler crabs are asymmetric consisting of a major and a minor type. The homologous closer muscle in these claws are innervated by two excitatory axons which, because they elicit tonic closing of the dactyl with repetitive stimulation and have neuromuscular synapses which facilitate, are regarded as slow type axons. The fiber composition of the paired closer muscles is also similar, being of the slow type. These fibers show typically long sarcomere (10 μm) and A-band (6 μm) lengths, light staining for myofibrillar ATPase denoting low specific activity for this enzyme, but more intense and circumscribed staining for NADH-diaphorase denoting a robust oxidative capacity. However, the protein composition of the paired muscles varies not only in its myosin light chain and paramyosin but also in the expression of isoforms for myosin heavy chain and several regulatory proteins including troponin I, tropinin T, and tropomyosin. Apart from any subtle functional differences imparted by these protein isoforms, the paired closer muscles are homogenously slow in their innervation and fiber type composition.

INTRODUCTION

In the past two decades a number of studies describing the neuromuscular organization in the dimorphic claws of crustaceans have emerged (see reviews by Mellon, 1981; Govind, 1984); studies prompted largely by our intrinsic interest in the asymmetry of body parts in an otherwise bilaterally symmetric animal. These studies have explored the cellular nature of the asymmetry in the paired closer muscles. In lobsters and snapping shrimps, these muscles are composed of slow fibers in the major claw and a mixture of fast and slow fibers in the minor claw. Curiously, in the lobster *Homarus americanus,* the paired muscles and their claws are symmetrical on hatching and only during subsequent juvenile development become asymmetric in a random manner *i.e.,* the major cheliped can occur either on the right or left side (Emmel, 1908; Lang *et al.,* 1978). The paired claws in very young, immature fiddler crabs are symmetrical and remain so as paired minor claws in females but become differentiated into a major and a minor claw in males (Morgan, 1924; Yamaguchi, 1977). The fiddler crabs therefore provide another example, apart from the lobster, where the acquisition of asymmetry in homologous structures may be studied.

However, there is little information on the paired asymmetric claws of male fiddler crabs apart from the above-mentioned work on the acquisition of asymmetry and

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Received 17 December 1985; accepted 25 March 1986.

Abbreviations: ATP, Adenosine triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol, bis (β-aminoethyl ether); N,N,N, N-tetra-acetic acid; NADH, nicotineamide dinucleotide, PMSF, phenyl methylsulfanyl fluoride; SDS, sodium dodecyl sulfate.

the behavioral role in feeding, aggression, and courtship (Crane, 1975). Huxley (1932) [illegible] the unpublished observation of Ratcliffe that the segmental ganglia and nerve [illegible] of the major claw were twice the size of those of the minor claw. More recently hypertrophy of the somata of the motoneurons and hyperplasia of sensory axons to the major claw have been documented (Young and Govind, 1983). Finally, Rhodes (1977) compared the fiber composition of several homologous muscles from the thorax and proximal segments of the claws in several species of fiddler crabs. He did not examine the claw closer muscle. Since this is the largest muscle in the claw and housed in the largest and most elaborate segment of the cheliped, it has been an obvious target for the study of asymmetry in other crustaceans such as snapping shrimps (Mellon, 1981), hermit crabs (Stephens *et al.,* 1984), blue crabs (Govind and Blundon, 1985), and lobsters (Govind, 1984). Therefore from a comparative viewpoint it seemed desirable to study the paired closer muscles in male fiddler crabs particularly since these animals provide the most flamboyant example of claw asymmetry (with the major claw being up to 50 times larger than its minor counterpart) amongst crustaceans studied so far. In addition, the correlation of contractile protein composition and muscle fiber type has recently been investigated in a number of crustaceans (Costello and Govind, 1984, Quigley and Mellon, 1984, Mykles, 1985a, b). The present study reports on the excitatory innervation, fiber type composition, and muscle protein expression patterns of the claw closer muscle in the male fiddler crab, *Uca pugnax.*

MATERIALS AND METHODS

Adult male fiddler crabs (*Uca pugnax*) were collected during the summer from salt marshes around Falmouth, Massachussetts, and held in the laboratory in natural or artificial seawater at 20–24°C. They were fed frozen brine shrimp and fish *ad libitum.*

The motor innervation of the claw closer muscle was determined using electrophysiology for which the cheliped was removed from the animal and dissected in marine animal saline (Govind and Lang, 1981). The leg nerve was exposed in the carpus and stimulated with a pair of fine platinum wire electrodes or a suction electrode. The resulting activity in the closer muscle was recorded as myograms with a pair of copper wires inserted into the muscle through the exoskeleton and insulated except at the tips. Simultaneously, intracellular recordings from single muscle fibers were made using conventional glass microelectrodes. For this purpose small windows were cut in the propus to expose selected portions of the closer muscle. The experiments were made at 15–16°C.

The fiber composition of the closer muscle was determined in two different ways. The first was to measure the sarcomere and A-band lengths of muscle fibers. The closer muscle was exposed by removing the overlying opener muscle and exoskeleton and fixed in alcoholic Bouins fluid for 24 hours. During this period the dactyl was held in a half-opened position to simulate a resting state of the closer muscle. Following fixation, the closer muscle was cut into quarters and stored in 70% alcohol. Measurements of the average sarcomere length (SL) and A-band lengths in these fixed myofibrils were made according to techniques conventional to our laboratory (Govind *et al.,* 1977).

The second way to type fibers was through a histochemial demonstration of myofibrillar adenosinetriphosphatase (ATPase) activity. To do this, the thickened exoskeleton of the propus of the major claw was ground down to almost the hypodermis with a high speed hobby drill. The minor cheliped did not warrant such pre-treatment. Next, the entire claw was frozen, cut in 16 μm cross-sections, and stained for myofibrillar ATPase activity by techniques described elsewhere (Ogonowski *et al.,* 1980). In addition the oxidative capacity of muscle fibers was assessed by staining some sections for NADH diaphorase activity. This latter technique reveals the density of mitochondria which is the principal site of the oxidative capacity of the muscle fibers.

Protein composition of the two cheliped muscles was also compared using one-dimensional SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Closer muscles were dissected, frozen in liquid nitrogen, and shipped on dry ice to Charlottesville, Virginia, from the Marine Biological Laboratory in Woods Hole, Massachusetts. All protein purification procedures were done on ice. Whole muscle tissue samples were prepared by homogenizing frozen muscle (one major or 5–10 minor closer muscles) in 40 volumes of solution A (40 mM NaCl, 5 mM phosphate buffer, pH 7.2; 1.0 mM MgCl, 0.1 mM EGTA, 0.1 mM DTT, 0.5 mM PMSF, 0.18/ml leupeptin, 1.0/ml α-macroglobulin). Immediately after homogenization an equal volume of 2× gel sample buffer was added and the samples were boiled. Actomyosin was made by centrifuging muscle homogenates, washing with solution A two additional times, and resuspending the washed myofibrils in solution B (0.6 M KCl, 0.01 mM MgCl, 5 mM phosphate buffer, pH 7.2, 0.1 mM EGTA, 0.1 mM DTT) for 30 minutes. The high salt insoluble fraction was removed by centrifugation and the actomyosin precipitated by dialysis against solution A. Pelleted actomyosin was resuspended at 100 mg wet muscle weight/1 ml of gel sample buffer. Myosin samples were generated essentially as described by Chantler and Szent-Györgyi (1980). Final myosin pellets were resuspended in a sample buffer at 1 mg/ml.

Whole muscle, actomyosin, and myosin samples from major and minor claws were compared on 12.5% acrylamide gels or 4–5% gradient gels and visualized with Coomassie blue stain.

RESULTS

Excitatory innervation

Innervation to the closer muscle was determined by stimulating the leg nerve in the carpus and monitoring the resulting myogram. Two distinct electrical responses with different latencies were recorded (Fig. 1A) suggesting the presence of two excitor axons to the closer muscle. We refer to these as E1 and E2 axons with E1 giving rise to the shorter latency myogram than E2. Furthermore, intracellular recordings in single fibers confirmed the presence of two distinct excitatory junctional potentials (ejps) corresponding to the extracellulary recorded myogram (Fig. 1B). Thus excitatory innervation to the closer muscle in fiddler crabs conforms to the established brachyuran pattern of two axons (Wiersma, 1961). No attempt was made to determine the inhibitory innervation to the closer muscle.

Stimulation of each axon elicited no visible twitch contractions with single or twin pulses but produced slow (tonic) contractions with repetitive stimulation at 5 Hz and higher. On this basis alone, both axons can resemble the slow or tonic type axons characteristic of the slow closer muscle in other brachyurans such as crayfish and lobsters, where the two excitors are differentiated into a fast and slow type.

The ejps evoked by each of the two axons were examined principally among the dorsal fibers and a few, more deeply located fibers. The axons responded in a similar fashion in both major and minor chelipeds. Usually E1 evoked a relatively small ejp seen only with repetitive stimulation when it would attain an amplitude of 1–2 mV, (Fig. $1B_1$, C). The E2 axon, on the other hand, gave with a single pulse an ejp of approximately 2 mV. With twin pulse stimulation (6–8 ms interpulse interval) this

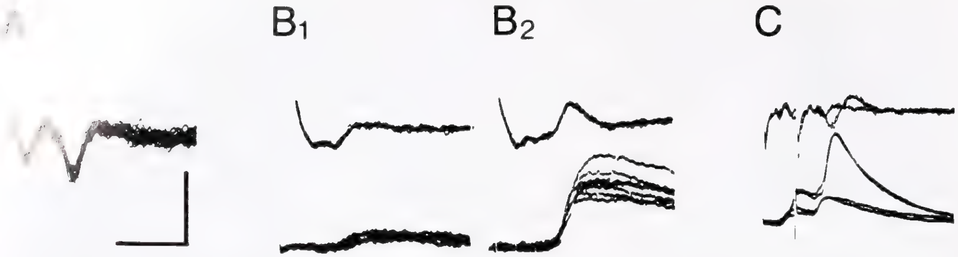

FIGURE 1. Excitatory innervation of closer muscle in minor claw. A; two distinct myograms in closer muscle with stimulation of cheliped nerve denoting innervation by two axons. $B_{1,2}$; myograms (upper traces) with different response times and their corresponding ejps (lower traces) in a single muscle fiber. Note the several sweeps in each ejp record showing little facilitation in B_1 but considerable facilitation in B_2. C; composite trace showing ejps (lower traces) and myograms (upper traces) to each of two axons stimulated separately and with twin pulses (separated by 6 ms) to show facilitation of ejps. Calibration, horizontal. A, B 8 ms, C 20 ms; vertical B, C 4mV.

ejp facilitated 3–5 fold in both claws (Fig. $1B_2$, C). In our limited sampling of fibers there did not appear to be any difference in the degree of synaptic facilitation between major and minor chelipeds. Synapses from both axons did not show any signs of fatigue when stimulated at 5 or 10 Hz for several minutes.

Fiber type composition of muscle

A well-established method for typing crustacean fast and slow fibers is by their characteristic sarcomere length (SL), with relatively short lengths (<4 μm) being indicative of fast-contracting fibers and longer lengths (>6 μm) of slow contracting fibers (Atwood, 1976). On this basis the closer muscles of the major and minor claw are composed of slow fibers having sarcomere lengths greater than 6 μm (Fig. 2). The muscle was divided into four areas and 30–40 measurements were made from each area revealing no differences among the areas. For the major claw muscle 159 measurements were made yielding a SL of 9.68 ± 1.09 and an A-band length of 6.15 ± 0.74 (X ± S.D.). For the minor claw muscle 100 measurements were made yielding a SL of 9.58 ± 1.45 and an A-band length of 6.12 ± 0.92. Since the A-band is less subject to variations in length than the sarcomere, the present results suggest a single population of long-sarcomere slow fibers in the closer muscles of both major and minor chelipeds. There are, however, several peaks in the distribution of A-band and sarcomere lengths in both muscles but whether these represent sub-types within the broad category of slow fibers is unknown. However, differentiation into sub-types also is suggested by histochemical evidence (see below). The distribution pattern in SL shown in Figure 2 was confirmed in three other animals where fewer measurements were taken.

The staining intensity for myofibrillar ATPase was uniform over the entire closer muscle as revealed in serial cross-sections of both minor and major claws (Fig. 3, upper row), in all six animals examined. This, combined with the fact that the fibers have long sarcomere lengths, strongly suggests that the closer muscle is of the slow variety. Further confirmation for this conclusion was obtained when myofibrillar ATPase activity was determined simultaneously in the closer muscles in the claws of fiddler crabs and in the walking legs of lobster, *Homarus americanus*. The closer muscle in the lobster has distinct fast and slow fiber populations (Govind *et al.*, 1981)

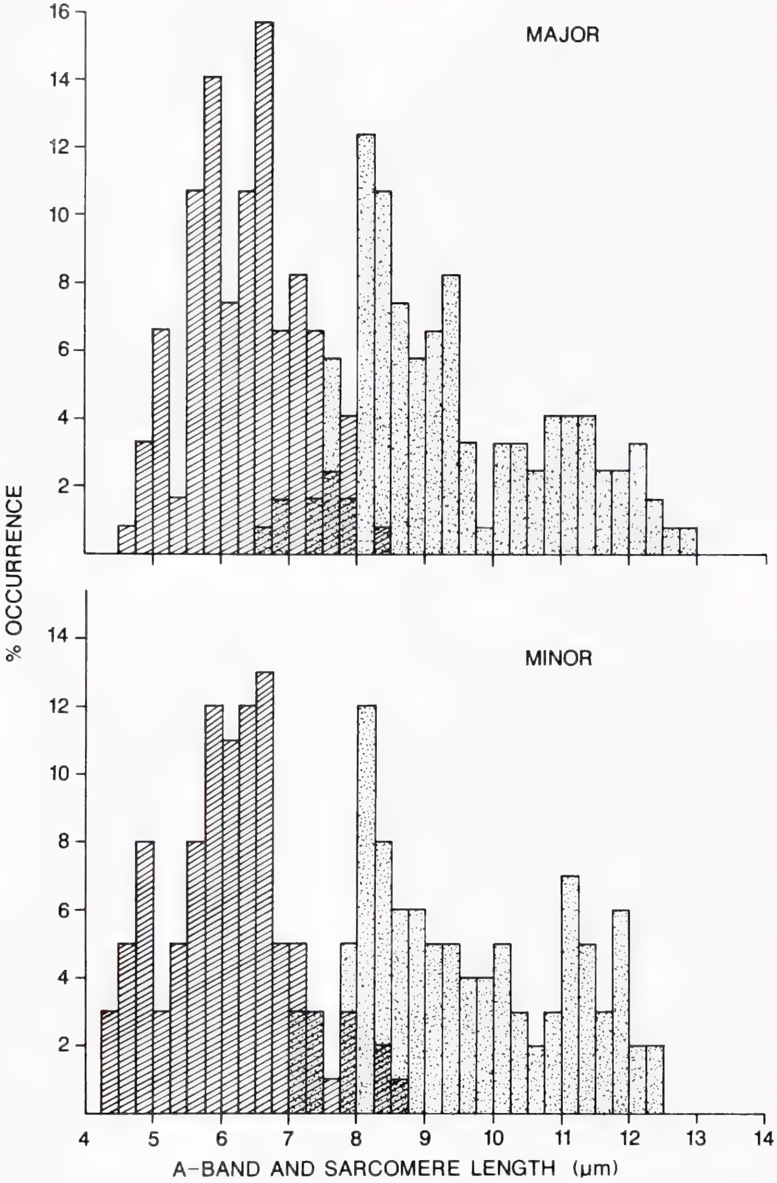

FIGURE 2. Percent distribution of closer muscle fibers with characteristic A-band (hatched) and sarcomere (stippled) lengths from a major and minor claw. Note the close similarity in distribution of these parameters between the two chelipeds. Number of measurements of A-band and sarcomere lengths is 159 and 100 for the major and minor claws respectively.

and we were able to judge that the staining intensity of the fiddler crab fibers resembled those of the lobster slow type. Thus, based on ATPase activity, the claw closer muscle in fiddler crabs is of the slow variety.

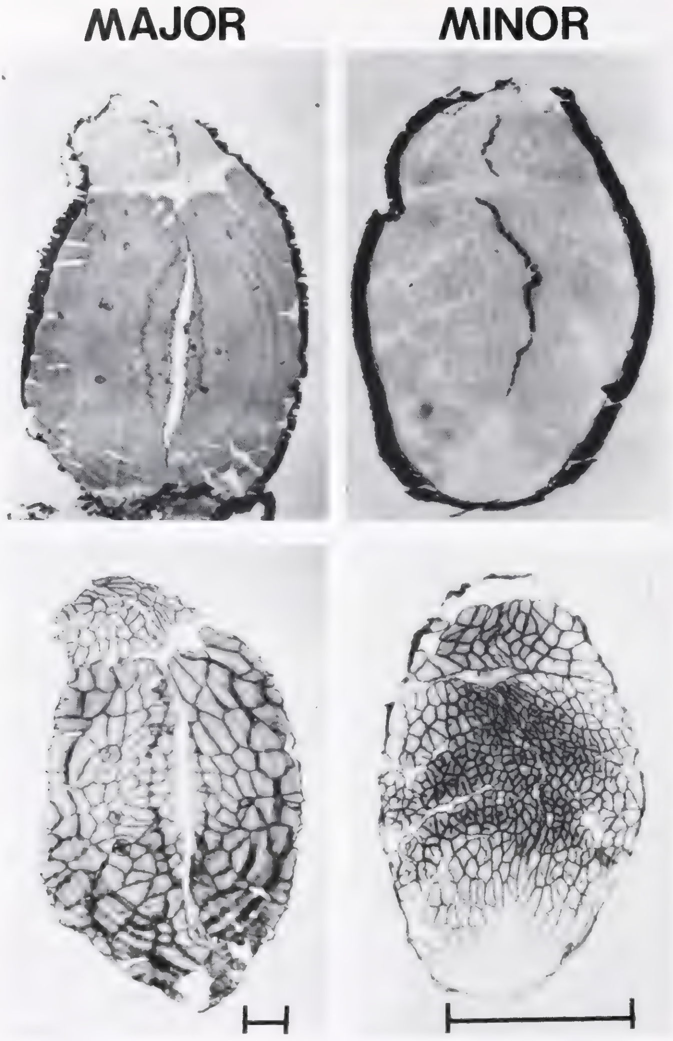

FIGURE 3. Representative cross-sections stained for myofibrillar ATPase (upper row) and NADH-diaphorase (lower row) from the mid region of the major and minor claws. The opener muscle is dorsally situated and smaller compared to the closer muscle which occupied most of the claw. Magnification 25×. Scale bar = 1mm.

However, subtle variation in staining intensity suggests differentiation of the slow fibers into sub-types. In the major claw muscle alone, a few distal fibers on either side of the tendon stain less intensely than the majority, suggesting a slower variety. But the most striking variation in the major claw is between the closer and opener muscle, with the latter staining much less intensely than the former (Fig. 3, upper row). The opener fibers appear to be of a much slower variety than the closer fibers. The ATPase staining intensity is similar between the opener and closer muscles in the minor claw. The opener muscle shows a regional distribution in staining intensity with the most lateral fibers staining slightly darker than the central ones. These subtle variations in ATPase activity suggest sub-types within the slow fiber category as has been previously shown in lobster muscle with histochemical (Kent and Govind, 1981) and biochemical (Mykles, 1985b) techniques.

To examine the oxidative capacity of the closer muscle fibers, frozen sections were stained for NADH diaphorase (Fig. 3, lower row) which is located principally in the mitochondria. These organelles tend to be most abundantly distributed around the periphery of muscle fibers and staining was restricted to this area in the major claw muscles. However some distal fibers in the major closer muscle revealed a higher density of mitochondria and as these were the same fibers which stained less intensely for ATPase, they represent a slower variety than the remainder. Similarly in the opener muscle of the minor claw, a much slower variety was recognized by the more densely stained profiles of fibers around the tendon compared to their counterparts at the lateral edges (Fig. 3, lower row). The closer muscle in the minor claw also revealed regional differentiation in oxidative capacity: fibers around the tendon showing a much higher density of mitochondria than those located more peripherally (Fig. 3, lower row). Clearly there is variation in both oxidative capacity and ATPase activity among the slow fibers of the closer and opener muscle suggesting further differentiation into sub-types within the slow category.

Identification of muscle protein isoforms

Until recently no identification of contractile protein isoforms had been reported in crustacean muscle. A number of investigators have now identified isoforms of the major contractile proteins by applying standard muscle protein purification procedures (Costello and Govind, 1984; Quigley and Mellon, 1984; Mykles, 1985a, b). We have taken this approach to compare contractile protein expression in the major and minor claw closer muscles to other crustacean muscles of known fiber type.

Figure 4 shows whole muscle, actomyosin, and myosin samples separated by SDS-polyacrylamide gel electrophoresis. Myosin light chain components have been identified by their presence in purified myosin samples. Both cheliped muscles contain a 15 K light chain (LC1) and 23 K doublet (LC2). We detect no difference in mobility of these components. This pattern is similar to that observed by Costello and Govind (1984) in lobster claw closer muscles. There is, however, an additional 21 K protein present in the minor claw myosin sample that is absent in the major claw (Fig. 4f). This protein can be detected in whole muscle samples prepared by homogenization in buffer containing protease inhibitors, followed by immediate boiling. Therefore this band is thought not to be a breakdown product and represents an intact component of the minor claw closer muscle.

A rather large number of putative troponin I (TnI) isoforms have been identified in crustacean muscles. Mykles (1985a) has resolved five TnI variants in different combinations in lobster muscles, and three TnI isoforms have been tentatively identified in *Alpheus*. In the region where TnI is expected to migrate we observed two bands in *Uca* actomyosin preparations. Both forms are present in each muscle (Fig. 4a–d), but in the major claw the faster-migrating, 25 K band predominates, whereas in the minor muscle the 26 K isotype is the major form. A similar distribution of TnI variants was reported in the claw closer muscles of *Homarus* (Costello and Govind, 1984). In that case it was concluded that these were fiber type specific isoforms.

Troponin T (TnT) and tropomyosin (Tm) subunits also exhibit heterogeneity in the cheliped muscles of *Uca*. Crustacean TnT and Tm migrate with apparent molecular

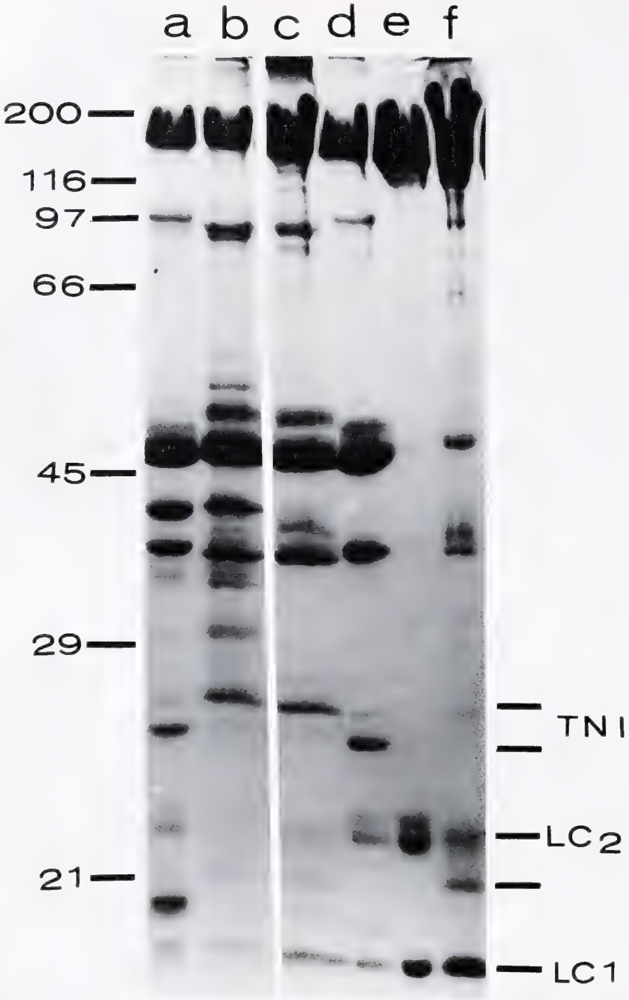

FIGURE 4. Whole muscle samples of major (a) and minor (b), actomyosin samples of minor (c) and major (d), and myosin samples of major (e) and minor (f) claw closer muscles, separated on a 12.5% acrylamide gel. Relative mobilities of markers of known molecular weight (×1000) are indicated on the left while major proteins of myosin light chain 1,2 (LC1,2) and troponin I (TN1) are indentified on the right.

weights near 52 K and 38 K respectively (Regenstein and Szent-Györgyi, 1975). In *Alpheus,* TnT expression in the asymmetric claws has been shown to be specific to claw type and not to fiber type *i.e.,* the pincer closer muscle which is made up of fast and slow fibers has a single TnT isotype, while the snapper muscle composed of all slow fibers, has another distinct form (Quigley and Mellon, 1984). The same pattern is observed in *Uca.* In Figure 5 *Alpheus* snapper and pincer closer muscle actomyosin samples were electrophoresed next to *Uca* closer actomyosin samples. With respect to TnT, the fiddler crab major claw appears similar to the pincer of *Alpheus,* since it contains a faster migrating subunit. The fiddler crab minor muscle TnT migrates more

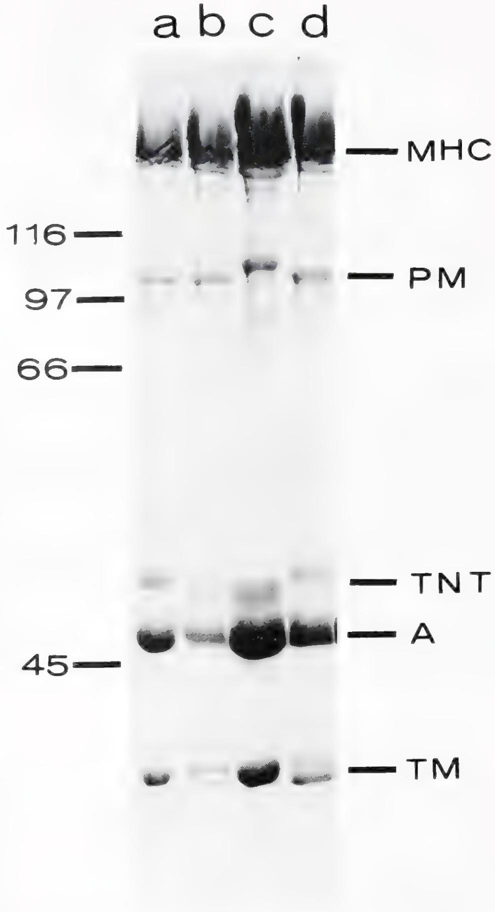

FIGURE 5. Actomyosin samples from the snapper (a) and pincer (b) cheliped closer muscles of *Alpheus* compared to those from the major (c) and minor (d) muscles of *Uca*. Samples were run on a 12.5% acrylamide gel for an extended period (8 h) to increase resolution in the 30–60 K range. Major proteins identified tentatively include myosin heavy chain (MHC), paramyosin (PM), troponin T (TNT), actin (A), and tropomyosin (TM).

slowly, similar to the snapper form in *Alpheus*. Interestingly the similarities are reversed for Tm expression. The fiddler crab minor closer muscle has a doublet at the Tm position, with each isoform present in apparently equimolar amounts. This is the same pattern observed in the snapping shrimp pincer claw. In the major claw of the fiddler crab and in the snapper claw of *Alpheus*, the larger subunit is found in excess (Fig. 5).

In *Homarus* (Mykles, 1985a) and *Alpheus* (Fig. 5) the expression of paramyosin isoforms has been correlated with muscle fiber type. We report differences in the protein banding patterns in claw muscles of *Uca* in regions where paramyosin is expected to migrate (Fig. 5). The minor muscle contains a faster-migrating component than the major muscle. Therefore it appears that crustacean slow muscles are not required to express the faster-migrating paramyosin isoforms.

The large size of the myosin heavy chain (MHC) has made detecting differences

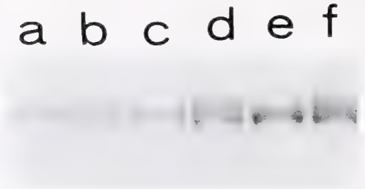

FIGURE 6. Separation of increasing amounts of myosin heavy chain (MHC) isoforms from minor (a, c, e) and major (b, d, f) cheliped muscles in low percentage acrylamide gels. Four to five percent gradient gels were run with whole muscle samples. Only that region of the gels where MHC runs is shown since most major components had run off the gel. Loading series consisted of approximately 0.5, 1.0, and 2.0 g of MHC for each muscle.

with standard one-dimensional gel electrophoresis difficult. Recently, however, low percentage acrylamide gels have been used to resolve MHC isoforms (Carraro and Catani, 1983). We have found that 4–5% gradient gels yield the best resolution. Figure 6 shows the MHC region of such a gel. A single MHC isoform is detected in the small claw, while two bands are present in the large claw sample. The samples loaded are whole muscle preparations so that differences are very likely not due to modifications introduced during purification. This represents the first demonstration of MHC isoforms in crustacean muscles. It is unclear what role this difference in MHC composition plays since no major differences in sarcomere length of myofibrillar ATPase activity have been observed in the claw muscles.

DISCUSSION

All of the properties of the closer muscle in the paired claws of the male fiddler crab explored in the present study are of representative slow type fiber. This is best exemplified by the muscle in which the fibers typically have long SL, low ATPase activity, and high oxidative capacity. Moreover, their protein composition resembles those of other crustacean slow muscle. Thus apart from differences in the isoforms of some contractile and regulatory control proteins, for which the functional significance is unknown, the closer muscle in the major claw is identical to its homolog in the minor claw.

Excitatory innervation of the closer muscle is similar on the two sides. Both muscles are innervated by slow axons which give rise to neuromuscular synapses which facilitate markedly and bring about tonic closing of the claw with repeated firing.

The similarities in neuromuscular properties between the paired muscles are somewhat surprising in view of striking asymmetry in the size and behavior of the claws. On the question of size, the major claw is some 30–50 times larger than the minor (Rhodes, 1977). Associated with this enlarged size of the major claw is a corresponding hypertrophy of the closer muscle, motoneuron somata, and hemiganglion, and a hyperplasia of sensory axons (Young and Govind, 1983). On the question of behavior, it is well-known that the minor claw is used for feeding while the major one is for courtship and defense (Crane, 1975). During feeding the closing action of the dactyl would be employed for gathering the substrate while other more basal segments of the claw would move the claw to the mouth where the substrate is deposited. These small basal segments would bring about the characteristic waving motion of the major claw during courtship. The closing action of the major claw would be used more in defense *e.g.*, arresting an intruder. Closing of the dactyl does not have to be unusually rapid in either of these feeding or defense situations. Consequently it is not surprising to find a slow type neuromuscular system in both the major and minor claws.

The degree to which homologous neuromuscular systems diverge in the dimorphic claws of crustaceans varies considerably. The present study of the paired closer muscles in male fiddler crabs shows little if any divergence except for some protein isoforms. In comparison the neuromuscular systems in the dimorphic claws of blue crabs *Callinectes sapidus*, are slightly more divergent (Govind and Blundon, 1985). Although the fiber composition of the paired closer muscles is of the slow type, the motor axons to the minor claw are effective at a lower frequency of firing than their homologs in the major claw. Moreover the excitatory axons to the closer muscles are differentiated into fast and slow types unlike those in the fiddler crab where both axons are of the slow type. Divergence of not only the motor axons but of the closer muscles is seen in hermit crabs (Stephens *et al.*, 1984), snapping shrimps (Stephens and Mellon, 1979; Stephens *et al.*, 1983), and lobsters (Lang *et al.*, 1977; Ogonowski *et al.*, 1980). In all of these species, the major closer muscle is composed entirely of slow fibers while the minor muscles has a mixture of fast and slow fibers. The percentage of fast fibers in the minor closer muscle is approximately 20–40% in snapping shrimps (Govind *et al.*, 1986), 40–50% in hermit crabs (calculated from data on sarcomere lengths provided by Stephens *et al.*, 1984), and 60–80% in lobsters (Govind, 1984). These figures show a tendency toward differentiating a largely fast neuromuscular system in the closer muscle of the minor claw in contrast to the major claw where it is entirely slow. Consequently the paired homologous neuromuscular systems of the claw closer muscles diverge from a symmetric state in blue crabs to an almost completely asymmetric state in lobsters.

Finally, the identification of protein isoforms in the closer muscles of fiddler crabs warrants some comment. Vertebrate muscle has been shown to contain fast and slow fiber type specific isoforms for most of the major contractile proteins (Jolesz and Sreter, 1981). Costello and Govind (1984) reported a correlation of isoform expression and fiber type in the claw closer muscles of the lobster. However these were the only two muscles examined. Mykles (1985a) subsequently reported, in a study of contractile protein expression in six lobster muscles, that protein composition of a muscle is not simply correlated with fiber types. This observation was also made when isotypes in homogenous slow, homogenous fast, and mixed muscles of *Alpheus* were compared (Quigley and Mellon, 1984). We have extended these findings to show that the homogenous slow claw closer muscles (as judged by morphology, physiology, and histochemistry) of *Uca* are heterogenous for a number of contractile protein isoforms. These include TnT, TnI, Tm, and MHC.

The meaning of these contractile protein isoform differences, in the face of no observable differences in contractile properties, ATPase staining, or sarcomere length, raises the question of what function the various isoforms play. One possibility is that the observations of physiological, histochemical, and morphological properties fail to identify subtle but significant differences in muscle function served by these proteins. Alternatively, some examples of fiber type or muscle-specific protein expression may be the consequence of the organization of muscle genes into coordinately regulated sets (Davidson and Britten, 1973) which may ensure the proper development and assembly of a muscle. It is possible that the various patterns of contractile protein isoform expression seen in the asymmetric claw closer muscles of crustaceans results from the grouping of contractile proteins into sets of large or small claw specific components. Clearly a better understanding of crustacean muscle protein biochemistry and the regulatory mechanisms for determining their patterns of expression is needed to address this question.

ACKNOWLEDGMENTS

We thank DeF. Mellon, Jr. and Joanne Pearce for critical reviews of the manuscript, and Anoop Dev, Jill Dawson, and John Guchardi for technical assistance. This work was supported by a Post-doctoral Fellowship from the NSERC to K.M.M. and by Operating Grants from NSERC and MDAC to CKG.

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THE ORGANIC MATRIX FROM OYSTER SHELL AS A REGULATOR OF CALCIFICATION *IN VIVO*

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ABSTRACT

Exogenously supplied, C-14 labeled organic matrix from oyster shell inhibited spicule formation by embryos of the sea urchin, *Arbacia punctulata,* as measured by simultaneous incorporation of inorganic C-14. Analysis of isolated spicules showed that the matrix reached the site of crystal growth and became incorporated into crystals. Comparison of the amount of matrix in spicules to inhibitory levels for matrix in an *in vitro,* pH-stat crystallization assay showed that the matrix became incorporated into spicules in an amount sufficient to account for the observed *in vivo* inhibition. The matrix did not act as a general metabolic inhibitor, as measured by respirometry, but rather seemed to have only the specific effect on spicule formation. Calculated and measured values of natural levels of matrix in spicules and urchin tests matched reasonably well with experimentally determined levels that regulated the rate of crystallization *in vivo.* Overall, the results support the idea that matrix fulfills a direct, regulatory role in biomineralization.

INTRODUCTION

The organic matrices from biological minerals are viewed as complex materials which regulate the growth and morphology of the mineral crystalline components (Mann, 1983; Weiner *et al.,* 1983; Greenfield *et al.,* 1984). The capability of matrix to regulate crystal growth *in vivo* may be manifested *in vitro* as the observed matrix induced inhibition of crystal nucleation and growth and change in crystal morphology when various crystallization assays are employed (Wheeler *et al.,* 1981; Sikes and Wheeler, 1983; Wheeler and Sikes, 1984; Wilbur and Bernhardt, 1985). Presumably, in these assays, matrix directly alters crystallization by binding to nascent crystals, as is the case for various synthetic crystal growth inhibitors (Reddy and Nancollas, 1973; Pearce, 1981).

Extrapolation of the *in vitro* assays to biomineral formation has been supported by the finding of Wheeler and Sikes (1984) that $CaCO_3$ incorporation into the spicules of sea urchin embryos could be inhibited when the embryos were incubated with fractions of water-soluble organic matrix extracted from oysters. However, the site of action of the matrix molecules was unknown.

On one hand, it seemed possible that the oyster matrix may have acted at the sites of spicule crystallization. However, the matrix is composed of relatively large polymers that according to prior studies would fall within the range of 20,000 to 180,000 daltons in molecular weight (Crenshaw, 1972; Krampitz *et al.,* 1976; Wheeler *et al.,* 1981; Wheeler and Sikes, 1985; Wheeler *et al.,* 1986). In view of the sea urchin embryo's frequent impermeability to molecules as small as disaccharides (Chizak, 1975), it

Received 18 November 1985; accepted 25 March 1986.

seemed possible that the site of crystallization *in vivo* would be unobtainable to the matrix. That is, the spicule is shielded from the external environment by several cellular and membranous layers which might act as barriers to the movement of matrix from the external medium to the site of crystallization (Okazaki, 1975).

An alternative way that matrix might affect $CaCO_3$ deposition would be by interfering with metabolism in some way that would indirectly result in an inhibition of calcification itself. It is also possible, of course, that the inhibitory effect of oyster shell matrix on sea urchin spiculation results from the combined activity of both the direct and indirect mechanisms of action.

The purpose of this paper is to clarify the mechanisms by which exogenously supplied matrix inhibits spiculation in the sea urchin embryo. The results provide evidence that matrix can inhibit and thus possibly otherwise regulate $CaCO_3$ crystallization *in vivo* by specifically binding to biomineral.

MATERIALS AND METHODS

Sea urchin embryo culture

Specimens of *Arbacia punctulata* were collected by divers from the sea wall at the State Park near Panama City, Florida. Collections were made from October through April when these urchins are fertile. On the day of collection, the urchins were transported to the laboratory in an aerated cooler containing natural seawater at 17 to 24°C, then transferred to an 80-gallon aquarium containing artificial seawater (ASW, Aquarium Systems) at 20°C with a salinity matched to that of the natural seawater (specific gravity 1.021 to 1.025). The urchins were fed lettuce every day and could be maintained in a healthy condition in the aquarium for several months.

Cultures of urchin embryos were prepared using standard methods (Sikes *et al.*, 1981). Fertilization typically occurred in greater than 90% of the eggs, with plutei development after 3 days incubation at 20°C.

Isolation and radioisotopic labeling of matrix

The organic matrix from oyster shell was prepared as described elsewhere (Wheeler and Sikes, 1985; Wheeler *et al.*, 1986). Basically, this involved extracting powdered shell in a dialysis bag against 10% EDTA at pH 8.0 until the mineral was dissolved, with the organic matrix retained in the bag. The insoluble component of matrix was removed by centrifugation at 30,000 × *g* for 20 min. The soluble matrix was dialyzed and concentrated using a Millipore Minitan ultrafiltration system with a nominal molecular weight exclusion limit of 10,000 daltons. Next the matrix was fractionated by gel filtration chromatography on Sephacryl S-300 (Pharmacia). The material that eluted in a broad peak with a median molecular weight of about 20,000 to 40,000 daltons was used in the incorporation studies and is hereafter referred to as "matrix."

The matrix was labeled using the method of Rice and Means (1971) in which C-14 methyl groups were attached to the protein amino groups by reductive alkylation using approximately 2 μmoles of C-14 formaldehyde (ICN Radiochemicals, 40 mCi/mmol) per mg protein. The activity of matrix as determined by use of crystallization assays as well as the chromatographic profile of the matrix were unaffected by the chemical modification. The specific activity of the matrix for the *in vitro* studies was 2.90×10^6 DPM/mg but was increased for the *in vivo* studies to 6.74×10^6 DPM/mg protein by increasing the specific activity of the C-14 formaldehyde used during preparation of the labeled protein.

All protein weights were obtained using the Miller (1959) modification of the method of Lowry *et al.* (1951). Matrix was nearly unreactive to the Bradford reagent (Bradford, 1976).

Radioisotopic measurements

In vivo *$CaCO_3$ deposition.* Embryos were harvested by hand centrifugation after incubating for two days. By this time, they had reached prism stage when spicules are first becoming visible under polarized light. Embryos were resuspended in fresh, filtered ASW at about 2000 embryos per ml. In some experiments involving uptake of C-14 labeled matrix, the concentration of embryos was raised to 5000 per ml to increase the detectability of the isotope in the samples.

To measure $CaCO_3$ deposition, radioisotope was added at a nominal value of 1 μCi/ml of culture from a stock of dissolved inorganic C-14 (DIC-14) in distilled water at 1 mCi/ml (ICN Pharmaceuticals, 53 mCi/mmole). The incubation vessels were 50 ml Erlenmeyer flasks with rubber stoppers covered with parafilm. This suppressed exchange of radioisotopic DIC-14 with the atmosphere. The embryos were incubated in 12 ml at 20 to 22°C on a gyratory shaker table set at 90 rpm to ensure uniform suspension of the embryos in the incubation medium. At intervals during the first 5 hours, 0.5 ml samples of the cultures were collected onto glass fiber filters (Gelman A/E, 25 mm, 0.4 μm retention) under vacuum at 7 psi. In some experiments, the incubations were allowed to proceed for 24 hours in flasks with cotton stoppers until the embryos began to become inactive as determined by examining swimming and ciliary activity by light microscopy. The embryos in 10 μl droplets were counted using a binocular microscope, with at least 10 droplets counted per treatment.

Samples on filters were immediately rinsed with 10 ml of unlabeled ASW then placed on a tray in a fume hood to dry overnight. This promoted removal of unincorporated C-14. Next the samples were vortexed in 20 ml scintillation vials containing 10 ml of scintillation cocktail (Beckman MP), then counted using a Beckman 5801 liquid scintillation counting system.

Following initial radioisotopic counting, some samples were treated with 0.3 ml of 1.0 *N* HCl added directly into the scintillation fluid. This lowered the pH of the fluid so that any DIC-14 including that incorporated into $CaCO_3$ in the sample was converted to CO_2. The vials were left open in the fume hood and vented for at least 12 hours. Control curves showed that this interval was sufficient to allow greater than 99% of the $^{14}CO_2$ to be exchanged with the atmosphere (Dillaman and Ford, 1982). The samples were then recounted with the residual counts attributed to acid-stable, non-volatile organic components (Sikes *et al.*, 1980).

Incorporation of C-14 matrix in vitro. The pH-stat crystallization assay was used as described elsewhere (Wheeler and Sikes, 1984) to measure the effects and the incorporation of matrix during $CaCO_3$ formation *in vitro.* In short, these assays were conducted at 25°C in 25 ml of a vigorously stirred solution containing 500 m*M* NaCl, 10 m*M* KCl, and 10 m*M* DIC with an initial pH of 8.45 to 8.50. To initiate crystal growth, 125 μl of a 2 *M* $CaCl_2$ solution was added to give a concentration of 10 m*M* Ca. This lowered the pH to approximately 8.3. Following an induction period of 2 to 4 minutes, $CaCO_3$ starts to form. This results in a decline in the pH of the solution according to the overall reaction, $Ca^{2+} + HCO_3^- = CaCO_3 + H^+$. However, the pH of the crystal growth solution was held constant at pH 8.30 ± 0.02 by autotitration with microliter quantities of 0.5 *N* NaOH using a Metrohm pH-stat system (Model 655 Dosimat with a 1.0 ml piston burette and a Model 614 Impulsomat) attached to a pH meter (Beckman model 3500). The progress of crystal growth was then followed by the quantity of titrant added, which for the concentrations involved was essentially equivalent to the quantity of $CaCO_3$ formed.

C-14 matrix was added during experiments after crystals had formed in an amount equivalent to 25 μmoles of added titrant. The percent inhibition was determined by comparing the rates of crystal growth before and after the addition of matrix.

The incorporation of matrix into crystals was determined by periodically removing 1 ml aliquots from the growth medium and collecting the crystals onto cellulose triacetate filters (Gelman type GA-8, 25 mm diameter, 0.2 μm pore size). The crystals were dissolved from the surface of the filters by washing them twice with 2 ml of 0.1 *N* HCl. The entire 4 ml was added to 10 ml of Beckman EP scintillation fluid and counted. Corrections for adsorption of isotope to filters were not significant and were made by use of control filters without crystals.

Incorporation of C-14 matrix into spicules. After incubation of embryos in the presence of C-14 matrix, it was necessary to isolate the spicules from the embryos so that any matrix that may have become associated with the spicules could be detected. To do so unambiguously, the isolated spicules had to be absolutely free from attached cellular debris. Accordingly, we needed to develop a technique for obtaining clean spicules.

Following incubations in which C-14 matrix was added to the culture, embryos were harvested in the normal way. Then they were resuspended in a solution of 5.25% sodium hypochlorite in a test tube that was placed in a sonicator (14 watts, Electromation Components) for 15 minutes. Following this treatment, spicules could be separated from cellular remains by hand centrifugation. This procedure was repeated two more times, with the spicules finally resuspended in isopropyl alcohol. Visual examination at 450× revealed complete, well-formed spicules with no trace of cellular debris.

This treatment was selected after a number of milder methods had failed. For example, sonication in isopropyl alcohol, 1% and 5% Triton, 1% and 5% sodium dodecyl sulfate, 1% and 5% dimethyl sulfoxide all yielded well-formed spicules upon hand centrifugation to separate spicules from cellular debris. However, in every case, a fairly extensive amount of cellular debris remained, particularly in the more fenestrated portions of spicules. This problem was compounded by the fact that spicules grown in the presence of matrix were even more prone to retain cellular attachments following these treatments with mild solvents. On the other hand, treatment with 1 *N* NaOH not only removed the cellular debris but also rapidly led to disintegration of the spicules themselves.

Other workers had shown previously that $CaCO_3$ structures treated with strong oxidants would retain an internal organic matrix (Crenshaw, 1972; deJong *et al.*, 1976). Similarly, hypochlorite has been used by others to prepare clean spicules from sea urchins (Okazaki, 1975; Mintz *et al.*, 1981) without apparent loss of matrix (Benson *et al.*, 1986).

Following hypochlorite treatment, the spicules were washed 3 times in and then resuspended in 5 ml of 10^{-4} *M* NaOH. Dilute base was used for this purpose because washing in distilled water led to partial dissolution of the spicules. Next, aliquots of the spicule preparation were taken for assessment of radioactivity by liquid scintillation.

Respirometry

The method for measurement of O_2 consumption by the sea urchin embryos has been described elsewhere (Sikes *et al.*, 1981). The method involves the use of an O_2 electrode and a Plexiglas chamber designed for assessment of respiration by cellular suspensions in 3 ml. The electrode (Beckman, Ag/Au) was energized by an adaptor assembled according to the design of Estabrook (1967) and connected to a voltmeter which was read to the nearest 0.1 mV and recorded by strip chart. Calibration curves were prepared by removing all O_2 from saturated solution by addition of Na_2SO_3. Saturation values for O_2 in seawater were taken from Kester (1975). In addition, filtered ASW to be used in respirometry was allowed to equilibrate with the atmosphere for 1 hour with smooth magnetic stirring prior to use. Confirmation of the saturation values of O_2 in seawater was made by use of the Winkler titration (American Public Health Service, 1981) with good agreement with the values of Kester. The respirometer was stable ±1 to 2 mV per hour and full deflection from saturated to anoxic conditions was set at about 50 mV. Embryos were capable of completely depleting media of O_2.

Statistics

Linear regression analysis was performed according to Kleinbaum and Kupper (1978) including tests for parallelism and coincidence. Analysis of variance was used to determine significance of differences in the tabular results (Keppel, 1973).

RESULTS

Matrix inhibited spicule formation in *A. punctulata* embryos (Fig. 1). The incubation medium for this experiment included both DIC-14 and C-14 labeled matrix. This allowed for both the formation of spicules and the incorporation of matrix to be measured in the same experiments. In Figure 1, each point is based on three replicate samples per experiment with a total of three complete experiments (n = 9).

C-14 uptake was significantly suppressed in the presence of matrix ($P < 0.01$). However, the incorporation of acid-stable C-14 in the presence of matrix was significantly elevated relative to control values ($P < 0.01$), presumably due to incorporation

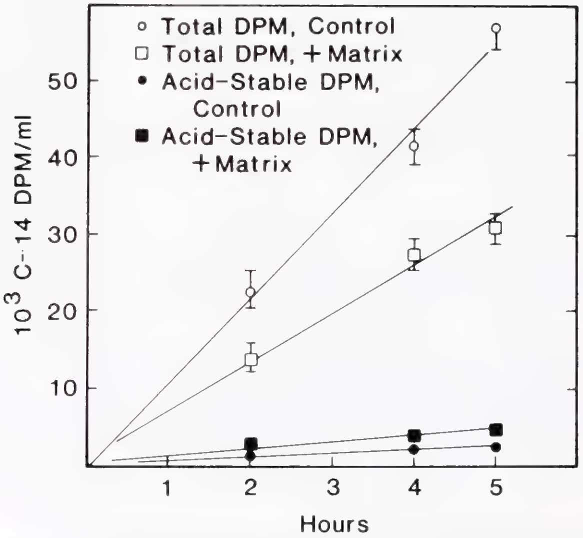

FIGURE 1. Incorporation of dissolved inorganic C-14 and C-14 labeled matrix by embryos of the sea urchin, *Arbacia punctulata*. There were 2800 ± 333 embryos/ml of medium (n = 10). The conditions for these experiments were the same as those listed in Table I.

of the C-14 labeled matrix into the embryos. Using the data of Figure 1, the amount of inorganic C-14 incorporation into spicules was determined as the difference between the total uptake and the acid-stable uptake. By computing this difference based on the slopes of the respective curves, a 48.5% inhibition of spiculation was observed over the first 5 hours of the incubation.

Final measurements were made after 24 hours (Table I). By this time, the urchins had reached the pluteus larva stage but did not develop further due to lack of food. By the end of the incubations, the relative contribution of inorganic C-14 to the acid-stable component became larger. This presumably occurred because the embryos approached saturation with the C-14 labeled matrix while the fixation of inorganic C-14 into organic material was more or less continuous. In any case, the ratio of C-14 in the acid-stable component compared to total DPM values was still higher in the experimental treatment.

Uptake of C-14 labeled matrix alone by urchin embryos was also measured (Table II) in three experiments. In these experiments a treatment at 2°C was used that has been shown to stop respiration and spiculation, but allows the embryos to resume normal activity upon warming (Sikes *et al.*, 1981). There was a temperature-dependent incorporation of C-14 labeled matrix by embryos during the 24 hour incubation ($P < 0.01$). Note also that a small but significant ($P < 0.01$) amount of the temperature-dependent incorporated radioactivity was associated with the spicules that were isolated by the hypochlorite method. In both cases, the temperature-dependent uptake suggested that the incorporation was dependent on metabolism.

In vitro crystallization also was suppressed in the presence of C-14 matrix (Fig. 2). An apparent decline in DPM/ml as the experiment progressed was due both to dilution by titrant and to settling of larger crystals even with vigorous stirring. Incorporation of matrix by crystals was corrected for the decline, which did not exceed 10% of initial values for DPM/ml. The concentration dependence of C-14 matrix on $CaCO_3$ crystallization is shown as redrawn chart recordings in Figure 3A. These results were replotted in Figure 3B to give the amount of matrix incorporated per μmole of NaOH titrated. The plots in Figures 2 and 3 are representative examples from families of curves which were replicated up to 10 times. Standard deviations in the amount of inhibition and the amount of matrix incorporated into crystals at particular levels of matrix were less than 10% of the values reported for these data.

Although doses up to 100 μg matrix/ml were supplied, there was no significant effect of the matrix on respiration by the embryos (Fig. 4). However, doses this high were essentially completely inhibitory to spicule formation. In these assays, the number

TABLE I

C-14 incorporation by embryos of the sea urchin, Arbacia punctulata, *that were incubated for 24 hours in medium containing both DIC-14 and C-14 labeled matrix*

	Total DPM $\times 10^{-3}$ (means ± SD)	Acid stable DPM $\times 10^{-3}$ (means ± SD)	μmole $CaCO_3$* per 10^4 embryos
Control embryos	765 ± 84.8	162 ± 32.4	0.766
Experimental embryos	499 ± 20.4	131 ± 93.5	0.467

* (2.2 μmoles DIC/ml) (total − acid-stable DPM/embryos) (DPM/ml) (2.72**).

** A correction factor to account for simultaneous incorporation of unlabeled respiratory CO_2 and DIC-14 by embryos (Sikes *et al.*, 1981).

There were 33,600 ± 4000 embryos (n = 10) in a total of 12 ml of medium. Radioactivity of the medium was 1.48×10^6 DPM/ml of which 6.93×10^4 DPM/ml were attributed to C-14 labeled matrix.

TABLE II

[illegible] of C-14 labeled matrix into whole embryos and isolated spicules [illegible] Arbacia punctulata

	Control 2°C (means ± SD)	Experimental 22°C (means ± SD)	μg matrix/ μmole $CaCO_3$*
C-14 matrix in whole embryos, DPM/10^4 embryos	3190 ± 107	8232 ± 747	11.0
C-14 matrix in isolated spicules, DPM/10^4 embryos	21.5 ± 0.953	113 ± 4.47	0.20

* (Expt'l − Control DPM/10^4 embs) (μg matrix/DPM) (10^4 embs/0.467 μmole $CaCO_3$).

There were 60.5 μg matrix/ml of medium giving a radioactivity of 59,600 ± 1110 DPM/min (n = 12). The culture contained 5020 ± 599 embryos/ml (n = 10) in 12 ml of total volume.

of embryos/ml was raised to 11,400 ± 1230 (n = 18) to allow a rapid measurement of O_2 consumption. This did not seem to affect the embryos adversely, in that the overall amount of O_2 consumption measured was 12.3 ± 0.569 nmoles O_2/1000 embryos/h (n = 9). This falls into the range of values of respiration by urchin embryos reported by other workers (Yanagisawa, 1975). The value for O_2 consumption was computed based on the slope of the curves over the first 15 minutes of an assay. As seen in Figure 4, after this time, the rate of O_2 consumption began to decrease, possibly due to damage to the embryos caused by the stirring needed to optimize the response of the oxygen electrode.

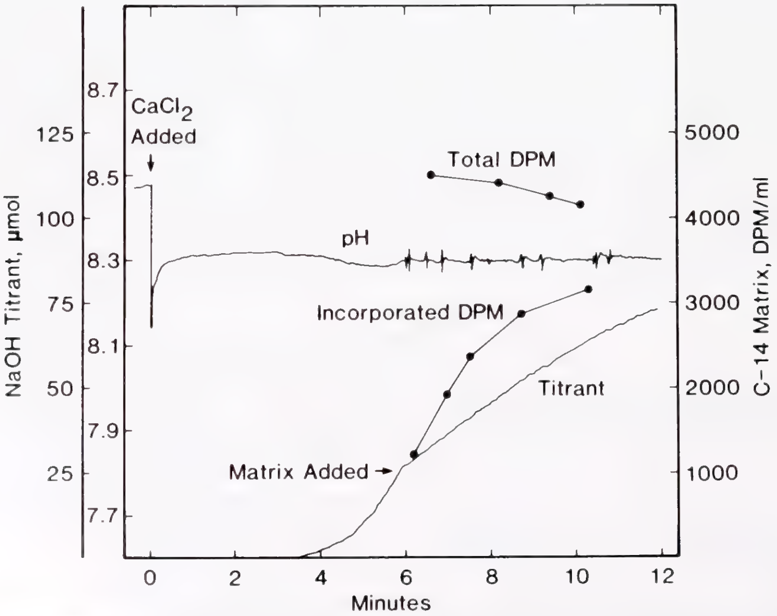

FIGURE [illegible] The incorporation of C-14 labeled matrix during $CaCO_3$ formation *in vitro* in the pH-stat crystallization assay. C-14 labeled matrix was added at 1.53 μg/ml (4.45 × 10^3 DPM/ml) after 25 μmoles of NaOH titrant had been delivered by autotitration.

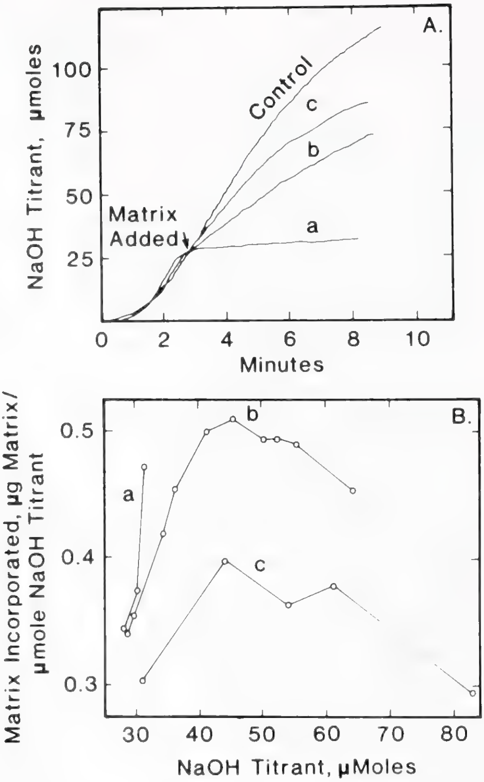

FIGURE 3. The effect of C-14 labeled matrix on $CaCO_3$ crystallization *in vitro* measured using the pH-stat assay. In part A, the control curve represents crystallization in the absence of matrix. Curve *a* shows a 97% inhibition of crystal growth when 1.84 µg matrix/ml was added; curve *b*, 53% inhibition at 1.53 µg matrix/ml; curve *c*, 23% inhibition at 1.22 µg matrix/ml. In part B, the amount of C-14 labeled matrix incorporated into the crystals grown during the experiments of part A is shown.

DISCUSSION

It is clear that the matrix from oyster shell, when added to the external medium, can reach the site of $CaCO_3$ crystallization during spicule formation by sea urchin embryos. This was evident from the experiments in which C-14 labeled matrix was detected in carefully isolated spicules from urchin embryos that had been grown in the presence of the labeled matrix (Table II). Spiculation was shown to be markedly suppressed in urchins treated with this amount of matrix (Fig. 1), indicating that the matrix did influence $CaCO_3$ deposition. In addition, the residual radioactivity of the spicules that we attributed to the presence of matrix was entirely acid-stable which eliminated the possibility that the C-14 may have been in the form of $Ca^{14}CO_3$, having become available as a source of inorganic carbon as respiratory CO_2 (Sikes *et al.*, 1981). These results also show that oyster shell matrix is relatively mobile in the sea urchin embryos despite the numerous intervening cellular and extracellular layers (Okazaki, 1975; Kingsley *et al.*, 1984).

To determine if the oyster matrix associated with spicules was sufficient to account for the observed inhibition of spiculation, the results *in vivo* can be compared to those of the pH-stat, *in vitro* experiments. This comparison seems reasonable because the

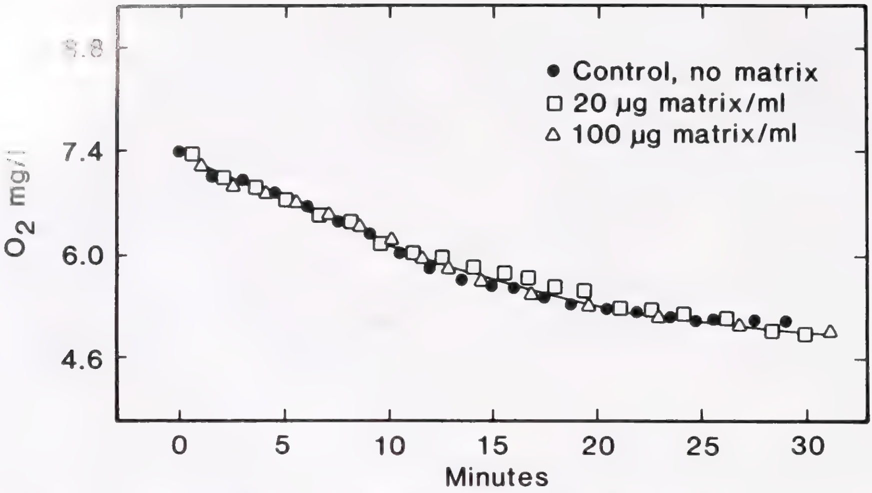

FIGURE 4. The effect of matrix on oxygen consumption by embryos of the sea urchin, *Arbacia punctulata*.

rates of crystal formation *in vitro* and *in vivo* can be similar. That is, based on the results of the radioisotopic incorporation (Fig. 1), urchins deposited 0.0166 μmoles of DIC-14 per ml (2800 embryos) per hour, or after correction for simultaneous incorporation of carbonate that originates as respiratory CO_2 (Table I), 0.0452 μmoles of $CaCO_3$ deposition per 2800 embryos per hour. By computing a spherical volume for the embryos using 50 μm as a typical radius based on microscopic examination using an ocular micrometer, each embryo would have a volume of 5.24×10^{-7} cm^3. Using this figure, we arrive at a value of 30.8 μmoles of $CaCO_3$ deposited per hour per cm^3 of embryos. For comparison, the rate of deposition in the pH-stat *in vitro* assay can be read from Figure 2 as 48 μmoles of $CaCO_3$ deposited per hour per cm^3 of solution.

Because of the similar rates of crystal formation, it seems reasonable to compare the concentrations of matrix required for inhibition *in vitro* to those that might occur *in vivo*. In the present study, the level of matrix incorporated into mineral that resulted in inhibition *in vitro* (0.4–0.5 μg per μmole $CaCO_3$) was somewhat higher than recorded *in vivo* (0.2 μg per μmole $CaCO_3$) which was consistent with the somewhat higher rate of deposition measured *in vitro* as compared to that *in vivo*. Further, it is important to bear in mind that because of simultaneous secretion of unlabeled matrix by embryos, the value of matrix measured by C-14 incorporation into spicules may be an underestimate of the total level of regulatory matrix to which growing crystals were exposed. Therefore, it can be tentatively concluded that the exogenously supplied C-14 matrix was associated with mineral in quantities sufficient to account for the observed inhibition of *in vivo* mineralization. In addition, because the matrix had no effect on respiration of the embryos, it seems unlikely that there was any indirect inhibitory effect of matrix on spicule formation.

At this point, the possibility that naturally occurring levels of spicular matrix may have regulatory effects similar to those seen with the C-14 matrix should be considered. Although oyster shell was the source of the matrix in the present study, spicular matrix also can inhibit spicule formation by urchin embryos (Swift *et al.*, 1986) and seems to be similar to oyster shell matrix in composition (Weiner *et al.*, 1983; Benson *et al.*, 1986). Thus it seems likely that the two matrices would elicit similar effects.

The value of 0.2 μg matrix incorporated per 100 μg (μmole) $CaCO_3$ measured during inhibition of spiculogenesis in the present study corresponds with the range of matrix (0.06–0.3% by weight) obtained for sea urchin tests (Pilkington, 1969; Weiner *et al.*, 1983; Swift *et al.*, 1986) and for spicules (approximately 0.1%) isolated in a manner similar to that of the present study (Benson *et al.*, 1986).

These values may include matrix which is not involved in regulatory functions and thus represent estimates for a maximum percent of regulatory matrix in mineral. For example, only 25% of urchin test matrix is a soluble fraction and thus similar to the C-14 matrix used in this study. If we accept that only the soluble fractions can regulate mineral formation; that is, as has been suggested, the insoluble fraction has a structural rather than regulatory role (Weiner, 1984), then the naturally occurring levels of soluble matrix might produce much less dramatic regulatory effects, especially with respect to controlling rate of crystallization. Of course, this has to be true at times during biomineralization, otherwise crystal growth might be terminated altogether.

In reality, the fraction or total proportion of matrix which can regulate growth is unknown. It may be that in some systems most matrix has regulatory capacity at some point between the time it is secreted and its final incorporation in mineral. Further, it is likely that the matrix is deployed *in vivo* such that different portions of the spicules or test receive relatively higher amounts of matrix at different times. This might occur if the organism was slowing or stopping the growth of crystals in one region while promoting crystallization in another.

Overall, this study provides experimental evidence that matrix levels in the approximate range of that found in biomineral can control rate of crystallization. Several authors have pointed out that biomineral growth must be limited, at least periodically (Watabe, 1965; Bevelander and Nakahara, 1969; Crenshaw and Ristedt, 1975; Weiner and Hood, 1975; Wheeler *et al.*, 1981; Borman *et al.*, 1982; Wheeler and Sikes, 1984). Certainly this also is true and it may be that one of the functions of some components of matrix is to stop the formation of a biomineral structure when needed. It is also possible that the inhibition of crystallization observed *in vitro* is not so much a reflection of matrix acting only to stop crystal growth but also to arrange crystal morphology. That is, soluble matrix as well as other inhibitors of crystallization can alter crystal morphology *in vitro* (Nancollas and Reddy, 1974; Kitano *et al.*, 1978; Rohm and Haas, 1983; Wheeler and Sikes, 1984). Presumably this occurs in response to the binding of the inhibitor to an existing crystal growth site with the result that new growth sites develop (Mann, 1983), changing the morphology of the crystal and ultimately resulting in biominerals of many varied forms.

The findings described herein do not contradict prior findings that matrix also may serve sometimes to nucleate or initiate crystal growth (Crenshaw and Ristedt, 1975; Sikes and Wheeler, 1982; Dillaman and Roer, 1984; Greenfield *et al.*, 1984; Wilbur and Manyak, 1984; Addadi and Weiner, 1985; Bernhardt *et al.*, 1986). Many authors have recognized the possibility that matrix may serve at least a dual function in regulation of biomineralization. Exactly how one material, the matrix, can fulfill the various functions assigned to it remains an interesting enigma (Wilbur, 1985).

ACKNOWLEDGMENTS

This work was supported by the Mississippi-Alabama Sea Grant Consortium, the Alabama Research Institute, and the South Carolina Sea Grant Program.

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CHANGES IN THE HEMOLYMPH ACID-BASE STATE OF THE SHORE CRAB, *CARCINUS MAENAS,* EXPOSED TO SIMULATED TIDEPOOL CONDITIONS

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ABSTRACT

High-tide and low-tide conditions were established in an artificial pool containing algae by alternately circulating seawater and leaving it stagnant for successive 6-h periods. Diurnal cyclical changes of water temperature and partial pressures of oxygen and carbon dioxide were almost the same as in natural tidepools. Hemolymph acid-base status (pH, carbon dioxide partial pressure, and bicarbonate concentration) was determined during day and night low-tide periods in crabs (*Carcinus maenas*) acclimated to artificial pool conditions. Except at water P_{O_2} below 20 Torr where the animals breathed air and developed a partially compensated respiratory acidosis, acid-base disturbances induced by changes of respiratory gases were much less than those observed in single-factor laboratory experiments, mainly because of counteracting influences of oxygen and carbon dioxide. This resulted in a decrease of hemolymph pH at increasing water temperature of -0.016 pH unit $\cdot$ $°C^{-1}$, consistent with the imidazole alphastat hypothesis.

INTRODUCTION

Large and rapid fluctuations of many environmental factors are known to occur in small water bodies left on the shore by the receding tide (Newell, 1979; Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983). In addition to variations of water temperature and salinity, the partial pressures and concentrations of respiratory gases, oxygen and carbon dioxide, may undergo conspicuous cyclic changes when animal and plant populations are present. During the day, photosynthesis usually surpasses respiratory activity, leading to an elevation of the water oxygen partial pressure (Pw_{O_2}) which may even be accentuated by a simultaneous increase of temperature. At the same time, carbon dioxide is depleted, water CO_2 partial pressure (Pw_{CO_2}) decreases, and water pH (pHw) markedly increases. When tidepools are isolated from the open sea at low tide during the night, the opposite changes of Pw_{O_2}, Pw_{CO_2}, and pHw are observed in the absence of photosynthesis. Thus, at each tidal emersion cycle, these aquatic habitats become hypoxic-hypercapnic at night and hyperoxic-hypocapnic during the day. In addition, most probably because large changes of pH favor either precipitation or dissolution of calcium carbonate, the total titratable alkalinity tends to decrease during the day and to increase at night (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983).

Received 6 December 1985; accepted 25 March 1986.

Abbreviations: α_{CO_2}: carbon dioxide solubility coefficient in blood; Cb_{CO_2}: total carbon dioxide concentration in blood; Pb_{CO_2}: carbon dioxide partial pressure in blood; pHb: blood pH; pHw: water pH; Pw_{O_2}, Pw_{CO_2}: oxygen and carbon dioxide partial pressures in water; TAw: water titration alkalinity.

All these factors acting separately are known to affect respiratory function and blood acid-base state of many aquatic animals (reviewed by Truchot, 1981a). For example, hypoxia induces an increase and hyperoxia a decrease of the ventilatory activity in fishes (Dejours, 1973), crustaceans (Jouve-Duhamel and Truchot, 1983), and annelids (Toulmond and Tchernigovtzeff, 1984). These ventilatory responses usually lead respectively to hypocapnic alkalosis and hypercapnic acidosis of the blood. Although ventilatory reactions to ambient CO_2 appear variable and their interpretation often controversial, numerous studies have unanimously demonstrated that an increased water P_{CO_2} induces a well-defined hypercapnic acidosis in aquatic animals. Acid-base responses to hypocapnic water, however, remain practically unknown, probably because of the difficulty of obtaining very low Pw_{CO_2} in laboratory conditions. Respiratory acidoses induced by either water hyperoxia or hypercapnia are usually compensated metabolically with an increase of the blood bicarbonate concentration, but these compensations develop slowly, taking many hours or days to be completed.

Obviously, the conditions to which intertidal animals may be exposed at low tide in rockpools are much more complicated than those examined up to now in laboratory experiments where the effects of each factor are studied separately. So many relevant factors vary simultaneously in natural tidepool conditions that the resulting changes of blood acid-base state are almost impossible to predict. The information available from laboratory studies is thus of limited significance in assessing physiological adaptation to this particular environment. In terms of survival value, the adaptational meaning of any physiological response to environmental changes can only be inferred from data obtained in ecologically realistic conditions.

According to the imidazole alphastat theory (Reeves, 1972, 1977), maintenance of an appropriate acid-base state in extra- and intracellular fluids is considered to be important in stabilizing macromolecular structure and function (Somero, 1981; White and Somero, 1982). This requires not only a constant pH at constant body temperature but also a temperature-induced change of pH of approximately -0.017 pH unit $\cdot$ $°C^{-1}$. Although many experimental data support this theory in various ectothermic animals, its general validity remains controversial. The required pH-temperature slope is not always found, particularly in aquatic animals (reviewed by Truchot, 1981a), and this failure often has been ascribed to a marked sensitivity of the acid-base status of water-breathers to environmental conditions. Indeed, by separately manipulating various ambient factors in water at constant temperature, it is very easy to induce large and durable changes of blood pH without apparent harmful effects to the animals (see Dejours and Armand, 1982). Despite suggesting a wide tolerance of aquatic species to extracellular acid-base disturbances, such experiments tell us nothing about actual acid-base variations in animals subjected to simultaneous changes of temperature and many other factors in their natural environment.

The shore crab, *Carcinus maenas,* a mainly intertidal species on west European coasts, is often retained at low tide in residual water bodies on the shore. Much information has been previously acquired in the laboratory on the effects of various environmental factors on hemolymph acid-base state in this animal (Truchot, 1973a, b; 1975a, b; 1978; 1981b; 1984). In addition, changes of ventilatory activity have been directly recorded at low tide in natural rockpools (Jouve-Duhamel and Truchot, 1983). The main objective of this work was to determine how these various effects combine to set the crab's acid-base balance in natural conditions and particularly to assess the validity of the alphastat hypothesis in a continuously changing aquatic environment. Using an artificial system faithfully reproducing tidepools conditions, I describe the pattern of hemolymph acid-base changes induced by simultaneous variations of many environmental factors in this habitat.

Materials and Methods

Observations were performed during the summer (June and July 1985) when environmental fluctuations in tidepools are the greatest (Morris and Taylor, 1983). Male shore crabs (*Carcinus maenas*), wet weight 30–50 g, collected near Arcachon, Southwest of France, were maintained at least one week before experiments in flowing seawater (temperature 19–21°C; salinity *ca.* 32‰) in the laboratory.

Artificial pool

Tidepool environmental conditions were established in an outdoor circular plastic tank of *ca.* 1.2 m diameter. Water volume was approximately 80 liter and the tilted bottom covered with a layer of gravel gave water depths between 3.5 and 9 cm, allowing the crabs to emerge spontaneously to breathe air, a behavior described by Taylor and Butler (1973). In the intertidal zone, natural rockpools are flooded at high tide and completely isolated from the open sea at low tide. Thus, to simulate high tide, water flowed through the tank at 1.1 liter $\cdot$ min^{-1} during a 6-h period which was followed by a low tide 6-h period when the water circulation was stopped. Algae, mainly *Enteromorpha* spp., *Ulva* sp., and some *Fucus,* were introduced to obtain a moderately loose cover at the water surface. They grew rapidly, attaining a total wet mass of 1.8 kg at the end of the experiment. Flourishing copepod and amphipod (mainly *Hyale* spp.) populations probably introduced with the algae were also observed.

Ten to twelve crabs were acclimated for at least two high and low tide cycles in the tank before sampling, which was performed various times after the beginning of a low tide period, during the day or at night. During rainy periods, excessive water dilution was prevented by a transparent plastic cover which was not used on sunny days to avoid undue heating.

Water and blood sampling

Once a crab was located in the artificial pool, temperature was first recorded and a water sample was then drawn into a 20 ml syringe close to the inhalent openings at the base of the walking legs. The crab was then rapidly grasped before it could run away and a prebranchial hemolymph sample was obtained in a 1-ml syringe by puncturing the base of a walking leg. Water and hemolymph samples were used only when the animal had remained completely motionless before the catch. Only one hemolymph sample was obtained on each crab.

Measurements and calculations

After thermal equilibration of the syringe in a water bath, water pH (pHw) and P_{O_2} (Pw_{O_2}) were measured with Radiometer electrodes at either 20, 22, or 25°C, according to the mean tank temperature expected for that particular day. The pHw values were corrected to the *in situ* temperature using temperature coefficients $\Delta pH/\Delta t$ determined in preliminary experiments on seawater samples kept anaerobically in closed syringes ($\Delta pHw/\Delta t = -0.013$ and -0.010 pH unit $\cdot$ °C^{-1} at pH values above and below 8.0, respectively). Pw_{O_2} values were similarly corrected using oxygen solubility coefficients at various temperatures and salinities (Riley and Skirrow, 1975). Water salinity was deduced from chloride concentration measured with a Radiometer CMT 10 Chloride Titrator. Total titratable alkalinity, TAw, was determined by Gran titration following a procedure outlined by Culberson *et al.* (1970). From pHw and TAw measurements, water P_{CO_2} (Pw_{CO_2}) was calculated as described by Truchot and Duhamel-Jouve (1980).

On each hemolymph sample, pH (pHb) was immediately measured at either 20, 22, or 25°C with a Radiometer G297/G2 pH microelectrode and was corrected to the *in situ* temperature using a $\Delta pHb/\Delta t$ coefficient of -0.0195 pH unit $\cdot\, °C^{-1}$, valid for *Carcinus* blood kept anaerobically in a closed syringe (Truchot, 1973a). Total hemolymph carbon dioxide concentration (Cb_{CO_2}) was measured by the method of Cameron (1971). From measured pHb and Cb_{CO_2}, and from the solubility coefficient α_{CO_2} and carbonic acid dissociation constants K'_1 and K'_2 at the prevailing temperature and salinity (Truchot, 1976a), the hemolymph P_{CO_2} (Pb_{CO_2}) and (bicarbonate + carbonate) concentration were calculated for each sample using the formulas:

$$Pb_{CO_2} = Cb_{CO_2}/\alpha_{CO_2}\left(1 + \frac{K'_1}{10^{-pH}} + \frac{K'_1K'_2}{(10^{-pH})^2}\right)$$

$$[HCO_3^- + CO_3^{2-}](meq \cdot L^{-1}) = \alpha_{CO_2} \cdot Pb_{CO_2}\left(\frac{K'_1}{10^{-pH}} + \frac{2K'_1K'_2}{(10^{-pH})^2}\right)$$

Unless otherwise stated, results are presented as mean values ± S.E.M. Statistically significant differences between mean values are reported at the $P < 0.05$ level.

RESULTS

General observations

Measurements were performed on 72 crabs during 6 daylight and 2 night experimental sessions. After switching to low tide conditions, the water usually became hyperoxic-hypocapnic in the daytime and hypoxic-hypercapnic at night. On one day session, however, the weather was very cloudy and both hypoxic and hyperoxic conditions were observed, depending on the time of the day.

The time-courses of the changes of the environmental factors were very similar to those recorded in natural rockpools in Roscoff, Brittany, France, during a previous study (Truchot and Duhamel-Jouve, 1980). Since water samples were taken only in the near vicinity of the crab to be subjected to hemolymph sampling, possible inhomogeneities in the water mass could not be documented. However, these inhomogeneities were most likely weak, since the various parameters measured on samples from different locations in the tank usually changed evenly with time. An example is shown in Figure 1 for a daylight and for a night low-tide period. During the day, Pw_{O_2} and pHw increased, and Pw_{CO_2} and TAw decreased; converse changes were observed at night. The only differences from previous observations concern the higher temperature range and the lower salinities, mainly because of the more southern location. On sunny days, temperature increased strongly up to 30°C, but decreased almost imperceptibly at night because air temperature was relatively high. Salinity changes were moderate, no more than 1‰ during an experimental session. A comparison of average extreme values recorded in the artificial pool after 4–6 h low tide with those observed in natural rockpools in Roscoff at the same season is shown in Table I. The striking similarity of the ranges indicates that tidepool conditions were successfully established in the artificial enclosure.

Crabs put in the tank exhibited first an exploratory behavior, walking around for some time and then remaining quietly hidden within the algal mat. They were agitated only at night when the water became hypoxic. At a Pw_{O_2} around 20 to 30 Torr, they typically exhibited an emersion behavior, raising the anterior border of the cephalothorax above the water level and taking air through the normally exhalent apertures,

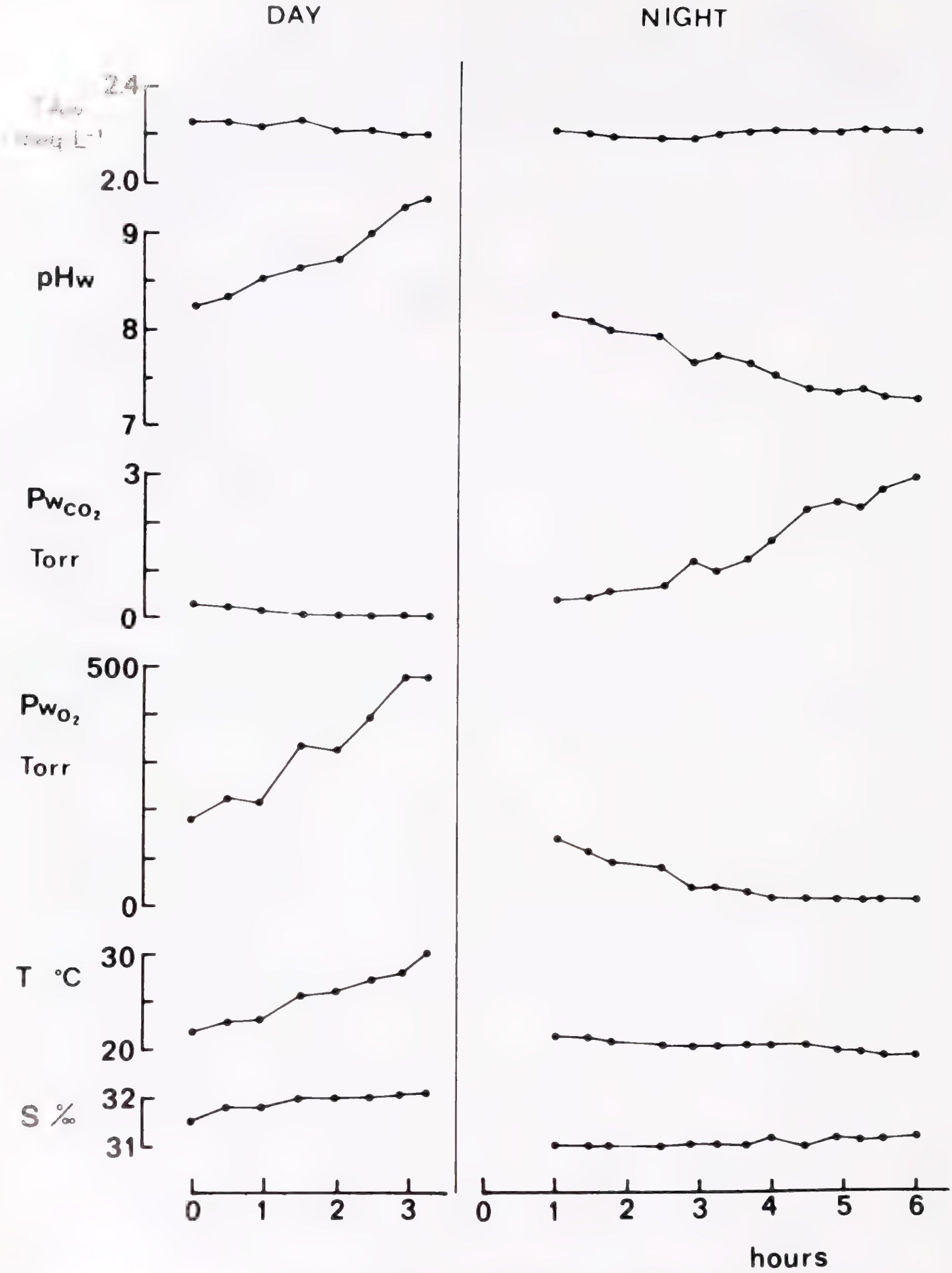

FIGURE 1. One example of the changes of temperature, salinity (S‰), pH, titration alkalinity (TAw), and partial pressures of oxygen (Pw$_{O_2}$) and carbon dioxide (Pw$_{CO_2}$) recorded in the artificial tidepool as a function of time elapsed after stopping water circulation during daytime or at night. Each data point at a given time corresponds to a water sample taken just before removing a crab from the tank for hemolymph sampling.

TABLE I

Average extreme values recorded after 4–6 h emersion in the artificial pool, compared to similar data at the same season (June–July) in natural rockpools on a more northern shore at Roscoff (from Truchot and Duhamel-Jouve, 1980)[1]

	Artificial pool (Arcachon)		Natural rockpools (Roscoff)	
	Night (n = 2)	Day (n = 6)	Night (n = 4)	Day (n = 4)
t (°C)	20.4 (—)	25.4 (2.2)	12.4 (0.2)	23.2 (0.6)
S (%O)	30.3 (—)	31.3 (0.3)	34.5 (0.1)	36.2 (0.2)
P_{o2} (Torr)	6.3 (—)	447 (48)	21.4 (12.0)	495 (28)
P_{co2} (Torr)	2.56 (—)	$2.17 \cdot 10^{-2}$ $(2.05 \cdot 10^{-2})$	1.77 (0.23)	$8.0 \cdot 10^{-4}$ $(2.8 \cdot 10^{-4})$
pH —	7.33 (—)	9.33 (0.20)	7.49 (0.05)	9.82 (0.09)
TA ($meq \cdot L^{-1}$)	2.25 (—)	1.92 (0.09)	2.46 (0.01)	2.05 (0.08)

[1] Mean values (S.E.M. in parentheses) from four different pools in Roscoff, and from two night and six daytime experimental sessions in the artificial pool.

as described by Taylor and Butler (1973). They generally reimmersed quickly when disturbed by an observer. Samples could be taken from these animals only when they were under water but frequent appearance of air bubbles indicated that most probably all immersed crabs had air in their gill chambers in these conditions.

Hemolymph acid-base values

Since the time-courses of the changes of the various environmental factors were different from day to day because of variable climatic conditions, presentation of the data as a function of the time elapsed from the beginning of a low-tide period would bear little meaning. Similarly, temperature changes were not reproducible from a given session to another. The conditions in this very variable environment are best described by the changes of respiratory gases and, for this reason, the acid-base values are presented according to the Pw_{O_2} range prevailing at the time of measurement. In Table II, seven Pw_{O_2} intervals, three in the hypoxic region, one normoxic, between 100 and 200 Torr, and three in the hyperoxic region are given. Except for the one below 20 Torr, corresponding to crabs predominantly breathing air, these classes are arbitrarily defined, but other limits do not modify the general pattern observed.

In moderately hyperoxic conditions, mean blood pH tended to decrease, albeit weakly, and remained steady or even increased when the water became strongly hyperoxic. Despite very low water P_{CO_2}, this tendency corresponded to Pb_{CO_2} values significantly elevated compared to those found in the normoxic range. Conversely, blood pH showed a trend to increase in the hypoxic range, at least down to 20 Torr

TABLE II

Mean hemolymph acid-base values for crabs Carcinus maenas *exposed to natural variations of environmental variables in an artificial tidepool during simulated low tide (°)*

	Pw_{O_2} range (Torr)						
	<20	20–50	50–100	100–200	200–350	350–500	>500
n	11	6	10	7	10	24	4
Mean Pw_{O_2} (Torr)	11.1 (1.2)	37.1 (3.7)	77.4 (5.7)	135 (11)	286 (20)	448 (7)	533 (5)
Mean Pw_{CO_2} (Torr)	1.96 (0.20)	0.99 (0.08)	0.60 (0.04)	0.38 (0.05)	0.11 (0.02)	0.01 (0.002)	0.002 (0.001)
Mean pHw	7.46 (0.05)	7.72 (0.03)	7.92 (0.02)	8.11 (0.05)	8.55 (0.05)	9.29 (0.06)	9.57 (0.07)
Mean temp. (°C)	20.7 (0.4)	20.9 (0.5)	21.4 (0.3)	21.4 (0.2)	23.7 (0.7)	26.7 (0.5)	28.8 (0.3)
pHb	†7.711 (0.026)	7.843 (0.032)	7.778 (0.033)	7.776 (0.046)	7.746 (0.032)	*7.703 (0.015)	7.736 (0.027)
Pb_{CO_2} (Torr)	**4.82 (0.48)	2.00 (0.32)	1.84 (0.16)	1.71 (0.17)	**2.49 (0.20)	**2.42 (0.09)	*2.27 (0.10)
$[HCO_3^- + CO_3^{2-}]$ ($meq \cdot L^{-1}$)	**11.20 (1.25)	6.74 (1.59)	5.35 (0.86)	4.87 (0.90)	5.97 (0.38)	5.11 (0.22)	5.06 (0.13)

(°) Data classified by range of water oxygen tension recorded. S.E.M. in parentheses.

* Significantly different at the $P < 0.05$ (*) or $P < 0.01$ (**) levels from the values recorded in the normoxic range (Pw_{O_2} = 100–200 Torr).

† Significantly different ($P < 0.01$) from the pHb value recorded in the 20–50 Pw_{O_2} range.

Pw_{O_2}, but blood P_{CO_2} did not significantly change. (Bicarbonate + carbonate) concentration did not change significantly from 20–50 Torr to >500 Torr Pw_{O_2}, remaining around 5–6 $meq \cdot L^{-1}$.

At Pw_{O_2} values below 20 Torr, when the crabs were likely breathing air during the night low-tide period, there was a steep significant decrease of blood pH and a strong increase of Pb_{CO_2} up to 4.8 Torr. This typical respiratory acidosis was acompanied by a marked increase of the blood (bicarbonate + carbonate) concentration.

DISCUSSION

Even though an artificial enclosure was used as a simulated tidepool, present data can be considered as representative of responses occurring in natural pool conditions at low tide on the shore. The reconstituted habitat was maintained with growing seaweed and flourishing animal life during more than one month. Furthermore, time-courses and extreme values of the relevant environmental variables, mainly temperature, Pw_{O_2}, and Pw_{CO_2}, revealed important fluctuations which were almost the same as those recorded in natural rockpools at the same season on west European coasts (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983).

According to previously published laboratory data (Truchot, 1973a; 1975a, b), if such large changes of physicochemical factors had been considered separately, there would have been important acid-base disturbances in *Carcinus maenas.* The most striking finding in this study is the modest variation of pHb and Pb_{CO_2} recorded in near natural conditions. Except in very hypoxic water at night, mean blood pH and P_{CO_2} ranged respectively from 7.70 to 7.85 and from 1.7 to 2.4 Torr.

The most important factors to be considered in interpreting these acid-base changes are the variations of the temperature and of the partial pressures of oxygen and carbon dioxide in the water. Salinity, which affects acid-base balance in *Carcinus maenas* (Truchot, 1973b; 1981b), changed too little to matter. The day-time decrease of seawater titratable alkalinity and its increase at night were too small to be implicated (Truchot, 1984).

Although acid-base disturbances were considerably damped in this study compared to previous laboratory findings, some well-defined trends can be discerned. In field conditions, *Carcinus maenas* is known to hypoventilate its gills during the day in hyperoxic-hypocapnic water (Jouve-Duhamel and Truchot, 1983). Accordingly, a slight hypercapnic acidosis did occur here during low tide in the daytime. However this disturbance was very limited, probably because of the simultaneous progressive lowering of Pw_{CO_2}. One initial purpose of this work was the study of the acid-base state of crabs in extreme hypocapnic conditions, which could not be reproduced in the laboratory. That the animals could maintain near-normal blood pH and P_{CO_2} values in this situation was unexpected, but resulted simply from the concomitant hypoventilation in hyperoxia. In the hypoxic range, there was a trend to a blood alkalosis, reminiscent of the internal hypocapnia usually associated with the hyperventilatory reaction induced by ambient hypoxia. However, the present data show no sign of decreased blood P_{CO_2}, probably because the water P_{CO_2} concomitantly increased in natural conditions. On the whole, at least for crabs relying only on water breathing at Pw_{O_2} values above 20 Torr, the pattern observed may be mainly explained by the counteracting effects of ambient Pw_{O_2} and Pw_{CO_2} changes, resulting in relatively limited acid-base disturbances. This is illustrated in Figure 2, which compares blood pH and P_{CO_2} values of this study with those from previous laboratory experiments at variable

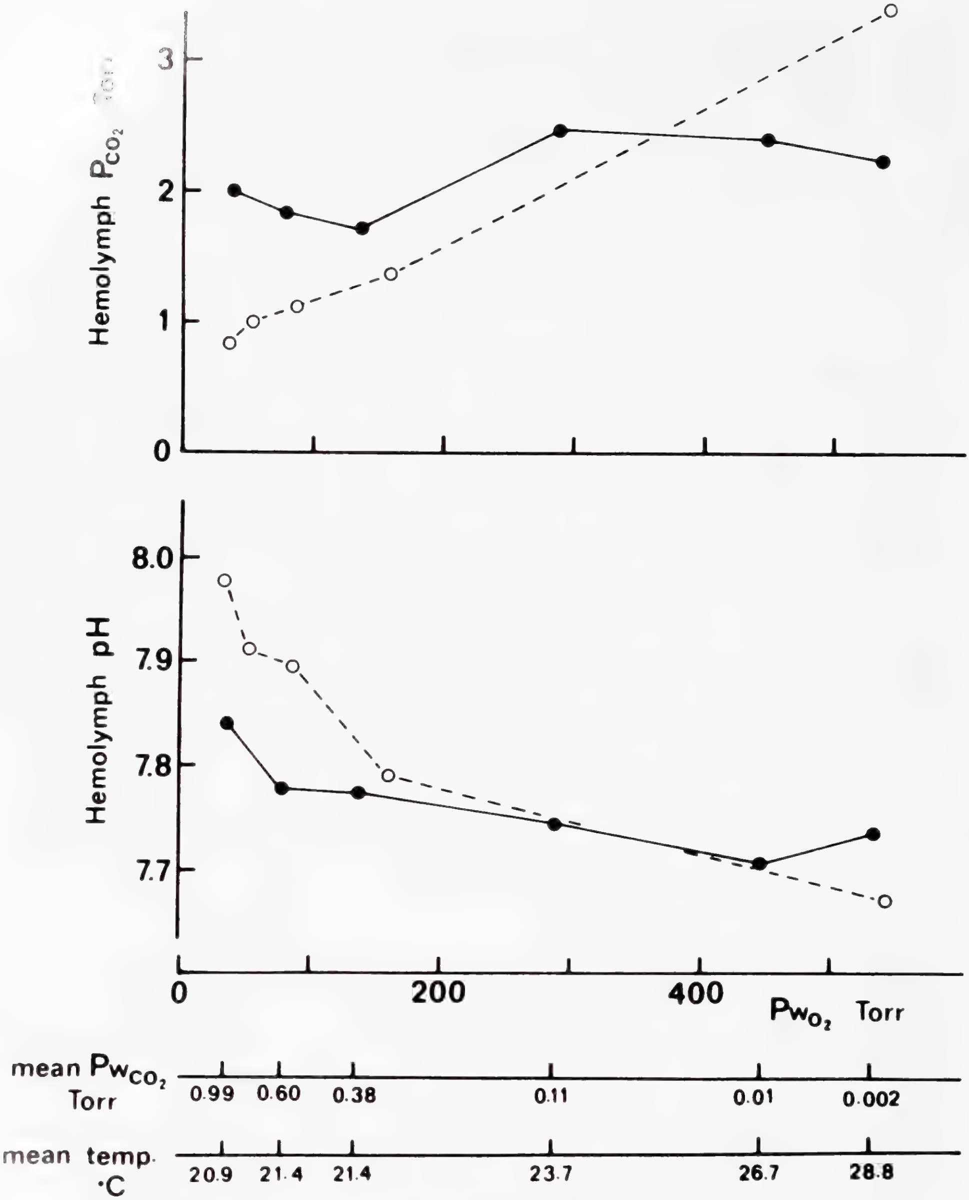

FIGURE 2. Mean hemolymph pH and P_{CO_2} values recorded in this study and in previous laboratory experiments on the crab *Carcinus maenas*. Data are plotted as a function of water P_{O_2} together with corresponding mean Pw_{CO_2} and temperature values recorded in the artificial pool. ● = data from this study at variable Pw_{CO_2} and temperature; ○ = data at variable Pw_{O_2} and constant temperature (17°C) and Pw_{CO_2} (about 0.3 Torr) (from Truchot, 1975a).

Pw_{O_2} but constant Pw_{CO_2} and temperature. It is apparent that the respiratory alkalosis usually recorded in normocapnic-hypoxic water is limited by the concomitant external hypercapnia observed in tidepool conditions. Similarly, in the hyperoxic range, the hypercapnic acidosis linked to the depressed ventilation remains moderate because the water becomes hypocapnic in the natural setting.

What happens in severe hypoxia at $Pw_{CO_2} < 20$ Torr requires special comment. The findings confirm previous observations in showing that partially emerged crabs taking air into their gill chamber rapidly developed a hypercapnic acidosis (Wheatly and Taylor, 1979). This particular respiratory mode is advantageous in terms of oxygen acquisition (Taylor and Butler, 1973; Taylor *et al.*, 1977) and present data indicate that the associated acid-base disturbance remains moderate. In near natural conditions, we observed the emersion response at relatively low ambient Pw_{O_2} ($\simeq$20 Torr at 20–21°C), much lower than in the laboratory observations of Taylor *et al.* (1977) (42 Torr at 17–18°C) and Wheatly and Taylor (1979) (51 Torr at 20°C). Whether this disagreement results from other factors, such as the Pw_{CO_2} level, remains to be studied. A related point is that crabs never did emigrate out of the water at high temperature, as observed by Taylor and Wheatly (1979). Since high water temperature was always associated with hyperoxia in the artificial pool, it may be that the emigration behavior is concerned more with oxygen acquisition than with heat avoidance.

Laboratory observations have shown that hypercapnic acidosis induced either by ambient hyperoxia, hypercapnia, or air breathing is partially compensated metabolically only after at least 10 hours in *Carcinus maenas* (Truchot, 1975a, b, c). Thus an important question is whether metabolic compensations occur in natural conditions, limiting acid-base disturbances during rapid environmental changes. The present data do not show any significant differences in the hemolymph bicarbonate concentrations for any Pw_{O_2} value above 20 Torr, suggesting that the relevant environmental factors change too rapidly to allow completion of appropriate compensations. However, the increase of bicarbonate concentration below $Pw_{O_2} = 20$ Torr appears too large to be accounted for by passive physico-chemical buffering in the hemolymph. In fact, the apparent buffer slope, $-\Delta[HCO_3^- + CO_3^{2-}]/\Delta pH$, calculated between mean observed values in the 20–50 and <20 Torr Pw_{O_2} ranges was 33.8 $meq \cdot L^{-1} \cdot (pH\ unit)^{-1}$, considerably higher than the usual *in vitro* buffering capacity of *Carcinus* hemolymph (Truchot, 1976b). This indicates that the hypercapnic acidosis is at least partially compensated metabolically, a process which may be more effective in *in situ* than in laboratory conditions, as is also suggested by observations on another crab, *Cancer productus* (De Fur *et al.*, 1983).

Present data must be now discussed within the framework of the imidazole alphastat theory of acid-base regulation in animals (Reeves, 1972; 1977). This theory contends that to stabilize protein function, intracellular pH should change with body temperature at a rate of -0.017 pH unit $\cdot$ $°C^{-1}$. It would obviously be advantageous for this intracellular homeostasis that extracellular pH changes with the same slope. That this is not always found in laboratory investigations on aquatic animals has been taken as evidence against the theory (for example Heisler, 1984). However, as stressed by White and Somero (1982), the thermal regimens customarily established in laboratory acclimation experiments can be very different from those encountered by animals in their natural environments. By design, laboratory experiments usually consider the effects of temperature changes with other factors held constant, particularly oxygen and carbon dioxide partial pressures. In the natural aquatic environment, this design mainly simulates seasonal temperature variations in large water masses, oceans, or lakes. Such changes are very much slower than those ordinarily imposed in laboratory experiments and they may allow for the development of many additional adaptive processes (White and Somero, 1982). By contrast, rapid temperature variations usually occur on an hourly or diurnal basis in smaller water bodies where they are most

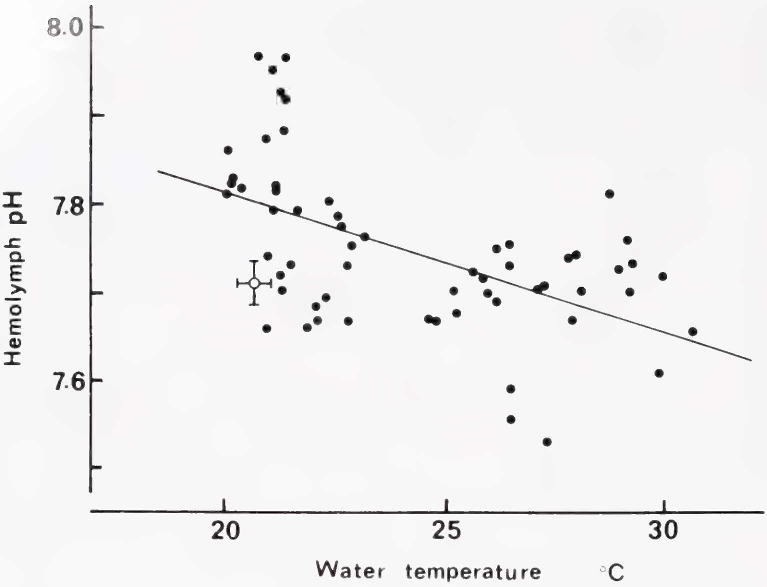

FIGURE 3. Individual pH values measured on 61 crabs *Carcinus maenas* breathing water in the artificial pool in low-tide conditions, plotted as a function of water temperature. Open symbol with error bars represents the mean pH value for 11 crabs breathing air at $Pw_{O_2} < 20$ Torr. Regression line calculated for values of crabs breathing water only. Correlation coefficient $r = -0.54$; $P < 0.001$.

frequently accompanied by large changes of oxygen and carbon dioxide partial pressures. This is the case in tidepools and the present data thus offer the opportunity to test the imidazole alphastat theory in ecologically realistic conditions.

Figure 3 shows a plot of individual pH data collected in this study as a function of temperature for crabs breathing water at $Pw_{O_2} > 20$ Torr. The scatter of these data is much larger than usually found in laboratory experiments. This is not surprising because environmental changes were not exactly the same from one low tide session to another and because different Pw_{O_2} and Pw_{CO_2} levels thus prevailed at a given temperature in the artificial pool. However, the pH-temperature slope calculated by linear regression amounts to -0.0157 pH unit $\cdot$ $°C^{-1}$ in the range 20 to 30°C with a highly significant correlation coefficient ($r = -0.54$; $P < 0.001$). The slope value fits the imidazole alphastat theory satisfactorily and approaches that required for a constant relative alkalinity (-0.0167 in the same temperature range). Practically the same slope was observed previously in laboratory experiments at constant water P_{O_2} and P_{CO_2} in crabs *Carcinus* acclimated several days at various temperatures, (Truchot, 1973a; Dejours *et al.*, 1985).

Customarily it is thought that pH-temperature adjustments in aquatic animals rely on changes of the blood bicarbonate concentration and thus require a relatively long acclimation period. The present data suggest that the same result can be attained rapidly in natural conditions when water P_{O_2} and P_{CO_2} simultaneously change with temperature. Whether this represents a particular adaptive pattern in intertidal rockpool animals awaits further studies. But, since no changes of hemolymph bicarbonate concentration are apparent in tidepool conditions, it may well be that the observed pattern results not from specific compensatory processes but simply from usual ventilatory responses to the concerted variations of P_{O_2} and P_{CO_2} occurring in this rapidly changing aquatic environment.

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CELL-SIZE DISTRIBUTIONS OF ZOOXANTHELLAE IN CULTURE AND SYMBIOSIS

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ABSTRACT

Zooxanthellae maintained in batch culture at 27°C with a 12 h:12 h light/dark cycle exhibited different cell-size distributions and morphology with growth phase. Mean cell diameter of cultured zooxanthellae isolated from *Zoanthus sociatus* increased with time of culture and growth phase from 6 μm (day 3) to 12.5 μm (day 11, late log-phase growth). Maximum specific growth rates ($\mu_{\max}$ $\log_e$ units) ranged from 0.3 to 0.4 day^{-1}. Within three hours after the onset of the light period on the third day of culture (early log-phase growth), large numbers of motile cells appeared. Subsequently, the dominant cell type was non-motile, vegetative, and possessed an accumulation body which increased in size with culture time. Zooxanthellae freshly isolated from a variety of hosts in their natural environment exhibited different cell-size distributions on an inter- and intra-host basis. Based on the laboratory results with cultured zooxanthellae, varying size distributions in fresh isolates of zooxanthellae from natural hosts may be explained by growth-related factors.

INTRODUCTION

Zooxanthellae (endozoic dinoflagellates) are the most prevalent algal symbionts of coral reef invertebrates (Taylor, 1974). Their role in host nutrition (Muscatine, 1980; D'Elia *et al.*, 1983) and the ecology of reef communities (Muscatine and Porter, 1977) is well studied. One encounters considerable debate in the literature about the systematics, distribution, and number of species and strains of zooxanthellae in existence: little consensus has yet emerged.

Recent work with cultured zooxanthellae indicates there are a variety of genetically different species (Blank and Trench, 1985) or strains that differ in morphology and cell diameter (Schoenberg and Trench, 1980a), mechanisms of photoadaptation (Chang *et al.*, 1983), patterns of motility (Fitt *et al.*, 1981; Lerch and Cook, 1984), and host selectivity (Schoenberg and Trench, 1980b).

Zooxanthellae in laboratory culture have a complex life history that alternates between motile gymnodinoid and non-motile coccoid stages exhibiting different cell size under certain conditions (Freudenthal, 1962; Trench, 1981). However, the relationship between cell-size distribution and morphology is poorly understood for zooxanthellae in culture, and there has been little attempt to relate the phase of growth of zooxanthellae to their morphological or physiological characteristics. In this paper, we characterize changes in cell size and morphology with growth phase for zooxanthellae in culture and examine natural variations in cell-size distributions for zooxanthellae freshly isolated from a variety of reef invertebrates.

Received 22 April 1985; accepted 4 February 1986.

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MATERIALS AND METHODS

[illegible] cultures

Gymnodinium microadriaticum isolated in Discovery Bay, Jamaica, from the Caribbean zoanthid *Zoanthus sociatus* was maintained in 100-ml batch cultures at 27°C, 12:12 h L:D period, 45 μEin $m^{-2}s^{-1}$ using the methods of Domotor and D'Elia (1984). Cultures were less than one year of age from the original isolation. To facilitate accurate determination of zooxanthellae division rate, cell size, and size distributions, cells in a 100-ml batch culture were scraped from flask surfaces with a rubber policeman and passed through 20-μm mesh Nitex screening by gentle agitation. This procedure separated clumped cells and produced a uniform suspension of algae but caused no damage to them; cells were intact and viable as verified by examination with bright-field microscopy.

Division and growth-rate determinations

Division and growth rates were determined in six trials (individual cultures) of *Gymnodinium microadriaticum*. Division rates were determined by monitoring increase with time in cell numbers using an Electrozone Celloscope particle counter (Particle Data, Inc., Elmhurst, Illinois) equipped with a 120-μm aperture tube. Growth rate was determined by monitoring changes in *in vivo* chlorophyll *a* fluorescence with time using a Turner Designs model 10 fluorometer (Mountain View, California) with CS-5-60 excitation and Wratten CS-2-64 emission filters.

Cell size distribution and morphology in cultured zooxanthellae

Cell sizes and their distributions were monitored at 1300 local time every 1–3 days over a 25-day period with the Electrozone computerized particle data system interfaced to a Digital PDP 8 computer. On several occasions bright-field microscopy was used to verify cell-size data obtained from the particle data system. Cells were examined for morphological changes using bright-field microscopy in conjunction with cell-size analysis.

Cell-size distribution in freshly isolated zooxanthellae

Zooxanthellae were isolated from a variety of reef corals, tridacnid clams, and zoanthids as described in D'Elia *et al.* (1983). Ten ml of freshly isolated zooxanthellae suspension were pipetted into plastic scintillation vials and fixed with an I_2-KI fixative (Utermöhl, 1958) for future analysis. Cell-size-distribution analysis was as described above for cultured zooxanthellae.

Control experiments

To determine if preservation with I_2-KI altered cell size, zooxanthellae isolated from several of the invertebrates sampled were examined using bright-field microscopy before and after addition of I_2-KI. Cultured zooxanthellae were examined using the particle data system before and after addition of I_2-KI. Comparisons of cell size before and after preservation indicated that cell sizes and size distribution were unaffected by the I_2-KI fixative. Examination of freshly isolated zooxanthellae with bright-field microscopy indicated that isolations were free of animal tissue fragments and bacteria that may have biased cell-size distribution determinations.

RESULTS

Division rates of cultured zooxanthellae

Maximum specific division rates of cultured zooxanthellae were determined on six different occasions using cell-count and *in vivo*-fluorescence techniques. Mean maximum division rates (μ_m in $\log_e$ units) from the two techniques were not significantly different (*t*-test, $n = 6$, $P < 0.05$), yielding means (±2 S.E.) of 0.35 ± 0.08 day^{-1} and 0.39 ± 0.18 day^{-1}, respectively.

Growth characteristics of cultured zooxanthellae

In culture, zooxanthellae isolated from *Zoanthus sociatus* were predominantly non-motile coccoid cells that clumped together and adhered to culture vessel surfaces, a characteristic noted by other workers who have maintained zooxanthellae in the laboratory (Freudenthal, 1962; Loeblich and Sherley, 1979). Gymnodinoid stages were observed predominantly in 3-day-old cultures corresponding to early log phase, with motility most noticeable about 3 h after the onset of the light period and lasting for approximately 0.5 h. These motility patterns were consistently observed for every subculturing of zooxanthellae, however, we emphasize that we made no systematic investigation of this phenomenon and such observations should be considered preliminary. Motile cells were positively phototactic and attached themselves to flask walls nearest to the light source.

Changes in cell size and morphology for cultured zooxanthellae

Figure 1 shows the size distribution of *Gymnodinium microadriaticum* with culture time and illustrates cell morphological types corresponding to peak cell sizes. There was a progressive increase in peak cell diameter from 6 μm (day 3) to 12.5 μm (day 11) with the greatest increase in the frequency of large cells occurring toward the end of log-growth phase. Cell diameter remained constant during days 11–25 of culture.

Cell size distribution from freshly isolated zooxanthellae

Primary and secondary peak cell sizes for zooxanthellae immediately isolated from invertebrate hosts sampled at different geographical locations are presented in Table I. The majority of the hosts sampled harbored two distinctly different size classes of zooxanthellae: a primary peak cell size of 8–14 μm and a secondary peak cell size of 5–7 μm (with the exception of *Montastrea* which had a primary peak cell size of 5 μm and a secondary peak cell size of 10 μm). Peak cell sizes (Table I) and actual size distributions (Fig. 2) of zooxanthellae isolated from the hosts sampled show that distinct differences in cell-size distributions exist on an inter- and intra-host basis. Replicate size distributions of zooxanthellae isolated from a coral (*Seriatopora*), a bivalve (*Tridacna*), and a zoanthid (*Zoanthus*) are variable in size range and frequency (Fig. 2).

DISCUSSION

Variations in cell morphology (*i.e.*, between life-cycle stages) are well known for zooxanthellae in culture, but changes in cell sizes and size distributions in relation to growth phase of a given stage have not been reported previously. Changes in cell diameter (from 6 μm to 12.5 μm) and cell-size distribution were observed particularly at the end of log growth with accompanying changes in cell morphology. We do not

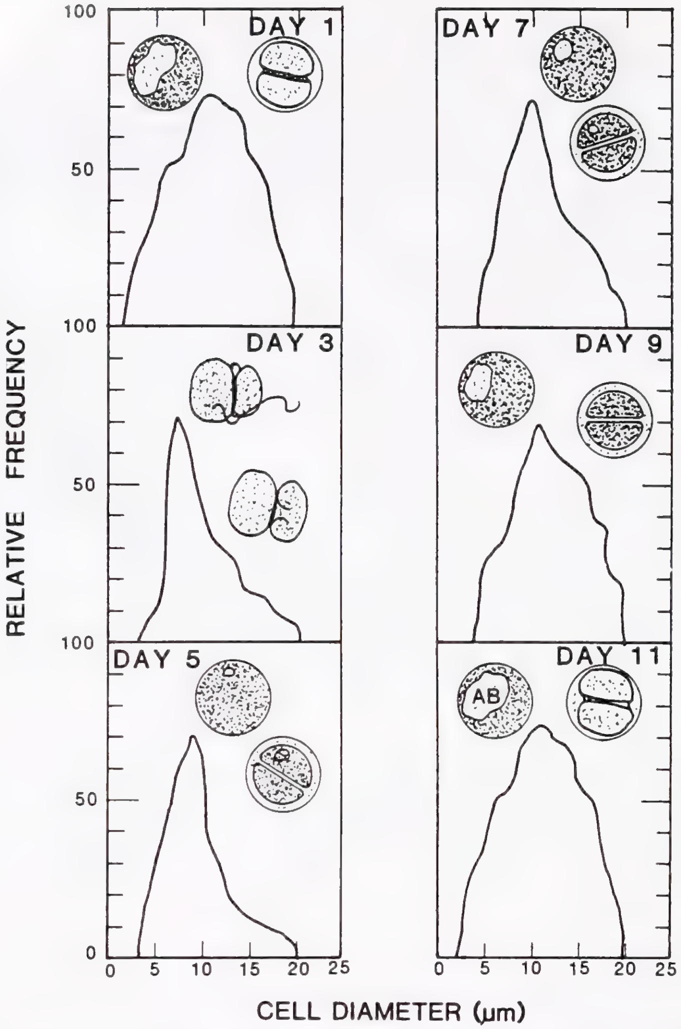

FIGURE 1. Size distribution of *Gymnodinium microadriaticum* with culture time. Observed cell morphological types corresponding to peak cell sizes are illustrated. Changes in cell morphology with culture time were: Day 1 = day of inoculation; aging vegetative cells and zoosporangia. Day 3 = gymnodinoid cells and dumbbell-shaped non-motile cells after dropping their flagella and attaching to solid surface; Days 5–9 = vegetative cells dividing to produce two daughter cells and vegetative cells aging with accumulation body enlarging as growth proceeds; Days 11–25 = aging vegetative cells with large accumulation bodies; some zoosporangia (AB = accumulation body). Two other cultures of zooxanthellae gave similar size distributions with culture time. There was no change in cell-size distribution during days 11–25.

know if cell-size and morphology for zooxanthellae in intact symbioses also vary with division rate, but we believe the possibility should be investigated.

Natural variations of cell-size distributions for zooxanthellae isolated from reef invertebrates occurred in this study on an inter- and intra-host basis. This could indicate (1) the presence of host-specific strains or species of zooxanthellae with different size

TABLE I

Primary and secondary peak cell sizes (based on percent of total cell number) of zooxanthellae isolated from a variety of reef invertebrates collected from a variety of geographical locations

Location of collection	Invertebrate host	Primary peak cell size (μm)	Secondary peak cell size (μm)
Cayos Arcas, Mexico	*Montastrea* sp.	5.2	10.2
Kaneohe Bay, Hawaii	*Zoanthus* sp.	8.9	
		8.5	
		7.5	
		$\bar{x} \pm$ S.E. = 8.30 ± 0.72	
Kaneohe Bay	*Pocillopora* sp.	13.8	7.0
Swains Reefs, Great Barrier Reef, Australia	*Seriatopora* sp.	8.9	5.5
		9.7	
		9.1	
		9.7	
		$\bar{x} \pm$ S.E. = 9.35 ± 0.41	
Swains Reefs	*Fungia* sp.	7.7	
Swains Reefs	*Acropora* sp.	11.5	5.2
Swains Reefs	*Tridacna* sp.	9.7	5.0
		12.3	
		10.2	
		$\bar{x} \pm$ S.E. = 10.73 ± 1.38	

and distribution characteristics, (2) variability of zooxanthellae growth and morphology in these symbioses, or (3) both. The presence of two distinct size classes of zooxanthellae in the majority of the invertebrates sampled is consistent with both possibilities, but at present we can only speculate that the high frequency of large cells observed in freshly isolated zooxanthellae correlates with late-log or stationary-phase growth.

Similarity between growth rates of cultured zooxanthellae calculated from cell counts and growth rates as determined by *in vivo* fluorescence measurements indicates that the two methods give comparable results. Maximum specific growth rates compare to those reported by other workers (*c.f.,* Loeblich and Sherley, 1979; Chang *et al.,* 1983).

Patterns of motility were similar to those observed by other workers (Fitt *et al.,* 1981; Lerch and Cook, 1984). Although motile stages of zooxanthellae may have been present throughout log phase, they were most noticeable on day three of early log-phase growth. Given the presence of large numbers of zoospores in stationary phase and the peak occurrence of motility in early log phase of culture, some algal or environmental factor apparently triggers the onset of motility.

The regulation of cell size, morphology, and growth/division rate of zooxanthellae in intact symbiosis is not well understood. Several possible factors or combinations of factors exist: (1) cell size, morphology, and growth rate are regulated by the zooxanthellae themselves, (2) these characteristics are regulated in some fashion by a host factor or nutrition, and (3) they are regulated by the environmental conditions in which the symbioses occur (reviewed by Cook, 1984).

If what we observed for cultured zooxanthellae in the laboratory applies to zooxanthellae in their hosts, varying morphological stages and cell sizes may correlate with varying growth rates, and factors related to growth and nutrition of the algae may be implicated. Future efforts seem warranted to determine the mechanisms that regulate zooxanthellae growth rate, cell size, and morphology by maintaining zooxanthellae

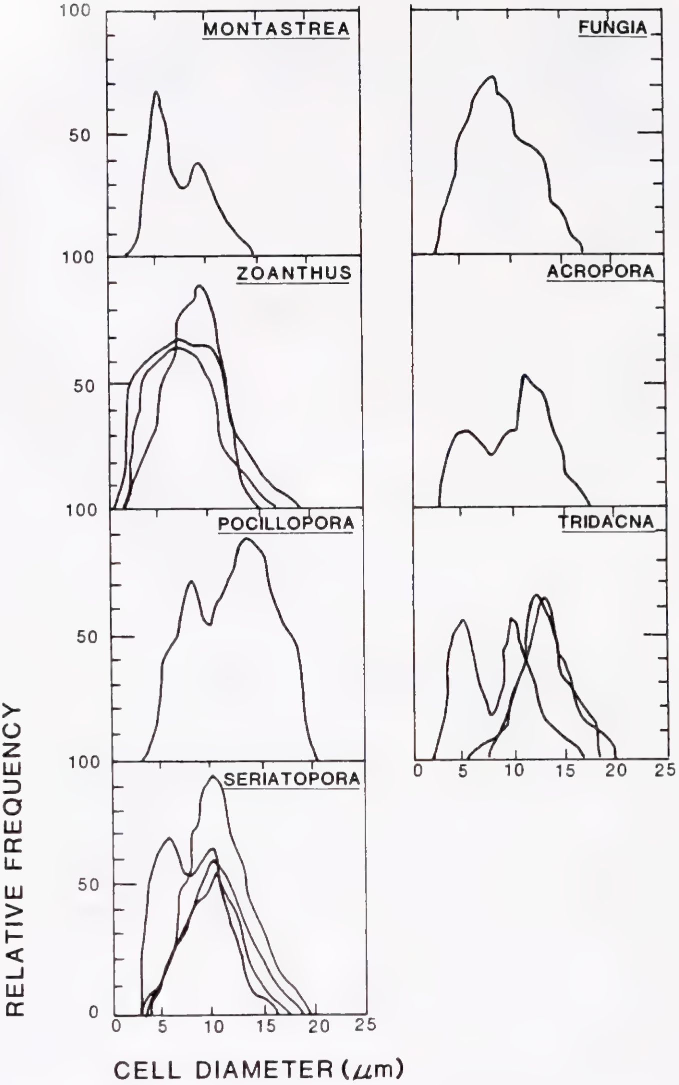

FIGURE 2. Cell-size distributions of zooxanthellae isolated from a variety of reef invertebrates collected from different locations. Replicate algal size distributions are presented for several of the hosts sampled.

under a variety of nutrient conditions (nutrient concentrations, rates of supply and sources) and light regimes (light quantity, quality, and photoperiod). Inasmuch as most studies employing cultured algae are done with clones maintained in the laboratory for months to years with progressive subculturing, an important question that needs addressing is whether zooxanthellae change nutritionally, physiologically, or morphologically with culture time from original isolation from the intact association through subsequent subculturing.

Clearly, more work with cultured zooxanthellae maintained under defined environmental conditions will help provide a better understanding of the mechanisms that regulate zooxanthellae cell size, morphology, and growth rate. The information obtained in such studies will be particularly useful if related to zooxanthellae in symbiosis.

ACKNOWLEDGMENTS

This research was supported in part by a Chesapeake Biological Laboratory Research Fellowship (to S. L. Domotor) and an NSF Grant OCE 78-08415 (to C. F. D'Elia). We thank J. G. Sanders and C. B. Cook for helpful comments on the manuscript, G. Fields for graphical assistance, and G. Canaday for typing the manuscript.

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MATE GUARDING IN AMPHIPODS: A ROLE FOR BROOD POUCH STIMULI

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ABSTRACT

In the amphipod, *Gammarus lawrencianus,* females carry fertilized eggs and occasionally brood juveniles in a ventral brood pouch. The male's decision to adopt and guard a female for mating purposes is influenced by the contents of the brood pouch. The presence of juveniles or recently fertilized eggs in the brood pouch inhibits the mate guarding decision; and an empty brood pouch facilitates it.

INTRODUCTION

In many animals, the male and female join together for a brief period of several days prior to mating. This phenomenon is called precopulatory mate guarding with reference to the observation that the male typically defends the female from other males during this period. This reproductive strategy has been of particular interest to sociobiologists and population biologists because of its implications for theories of sexual selection, and its potential contribution to our understanding of mating systems in general (Trivers, 1972; Parker, 1974; Wickler and Seibt, 1979; Grafen and Ridley, 1983).

Among invertebrates, precopulatory mate guarding is widespread in the major groups of Crustacea (Dunham, 1978; Atema and Cobb, 1980; Salmon, 1983). *Gammarus,* for example, is a large genus of amphipods that have adapted to almost every type of aquatic habitat in the world. In spite of this diversity, the reproductive strategies of these small crustaceans are surprisingly uniform. *Gammarus lawrencianus,* a euryhaline amphipod which is found in estuaries along the northeast coast of North America is a typical example. In this polygynous species, mating occurs immediately after each female molt. Following sperm transfer, the female releases eggs into a specialized ventral brood pouch where they are fertilized. The embryos develop in the brood pouch for a number of days and eventually hatch as juveniles that are released into the water column. This cycle is typically repeated every 9–12 days during the reproductive season.

Precopulatory mate guarding can be initiated at any time prior to the female molt. A highly stereotyped sequence of behavior is elicited when a free-swimming male comes into contact with an appropriate female. The male grabs her with his gnathapods and draws her close to his ventral surface in a position which places the longitudinal axes of their bodies at right angles to each other. In this position he actively palpates the female with various appendages including his antennules. After a period of palpation, the female is either rejected *or* rotated into the precopula position with the female underneath the body of the male and their body axes parallel. The pair swims together in this precopula position until the female molts and sperm transfer is com-

Received 15 July 1985; accepted 24 March 1986.

pleted, at which point they separate. Available evidence suggests that the females adopt a passive role throughout this process, and various laboratory and field observations indicate that the probability of finding a female in precopula with a male increases as she approaches the final stages of her molt cycle (Hartnoll and Smith, 1978; Birkhead and Clarkson, 1980; Hunte *et al.*, 1985; Dunham *et al.*, in press).

EXPERIMENT 1

Over the past few decades a considerable amount of research and speculation has attempted to identify a critical stimulus (or stimuli) which elicits precopulatory mate guarding in amphipods (Dunham, 1978, in press). The most viable hypothesis to date is that a pheromone released by the female mediates this process. However, several different investigators have questioned this assumption, (*cf.* Dunham, 1978; Hartnoll and Smith, 1980; Borowsky, 1984), and, at present, the specific factors which control the mate guarding decision have not yet been isolated.

Recently, some personal, informal observations of *G. lawrencianus* in a breeding culture suggested that the contents of the female's thoracic brood pouch might play a role in the male's guarding decision. First, during the palpation phase, the females are held in a position which permits the male to palpate this structure; second, the female is accepted or rejected after this palpation; and finally, close inspection of various females suggested that females with an empty brood pouch are accepted more quickly than those carrying juveniles. This first experiment was designed to provide a more formal test of these informal observations by directly comparing females with an empty brood pouch to those carrying juveniles to determine if there are behavioral differences in their mate guarding interactions.

Materials and methods

Subjects. A semi-naturalistic 20 l breeding culture of *G. lawrencianus* maintained at a field station on St. Margaret's Bay, Halifax County, Nova Scotia, was the source of all animals used in these experiments. Small stones and fucoids provided natural cover in the culture, and the animals were exposed to the natural day-night cycle occurring at this latitude from January to March. Water temperature varied from a low of 8°C at night to a daytime high of 18°C. Animals were demand-fed Tetra Marin fish food, small bits of mussel (*Mytilus edulis*), and dried squash flakes. Under these conditions, the average length of the female reproductive cycle (copulation to copulation) was 14.8 days with, on average, the last 5 days of each cycle spent in precopula with a male.

Procedure. Twenty-eight precopula male-female pairs were sampled from the breeding culture for observation. All observations were conducted between 1200 and 1400 hours at a water temperature of 15 ± 2°C. Prior to the observations each precopula pair was gently separated and placed in individual 50 ml containers of seawater. Following this brief period of isolation, both containers were emptied into a common third container (6 cm diameter) that served as a test arena where the animals could re-establish the mate guarding relationship. An observer maintained a real-time record of the number of contacts between the animals and the category and duration of each contact. Contacts were coded into two different categories: (a) contacts in which the male positioned the female for *palpation;* and (b) contacts in which the female was placed in the *precopula* position. Following each test, the female was examined under a microscope to determine the state of her brood pouch.

Results

Of the 28 pairs sampled from the breeding culture, 14 females had empty brood pouches, 12 carried recently hatched juveniles, and 2 pairs, damaged during separation, were excluded from the analysis.

The behavioral differences between these two different groups of females during the mate guarding assay are presented in Table I. These data make three important points. First, males take approximately the same amount of time to establish an initial palpation contact with either type of female (juveniles present *vs.* juveniles absent). Second, once a palpation contact is made, the time required to make the precopula decision is significantly shorter with females that have empty brood pouches ($t = 3.9$, $P < .005$). Third, mate guarding was eventually re-established in all pairs regardless of the brood pouch contents. Apparently, the presence of juveniles in the brood pouch does not alter the initial "attractiveness" of the female or prevent eventual mate guarding, but the juveniles do increase the time it takes the male to decide to guard the female.

EXPERIMENT 2

Changes in the contents of the female's brood pouch would be an adaptive stimulus upon which to base the precopulatory mate guarding decision. The presence or absence of embryos and the state of their development provides easily discriminated information about the time remaining before the next opportunity to copulate. Large, yolk-filled, recently fertilized eggs predict a long mate guarding investment, whereas an empty brood pouch signals a short investment time. However, there are other changes in the female which are perfectly correlated with changes in the brood pouch contents, and are also able to predict the time remaining until copulation. For example, changes in both molt physiology and ovary development are correlated with embryonic development in the brood pouch. Although the data from Experiment 1 suggest that brood pouch contents influence the mate guarding decision process, these other confounded changes may also be necessary and/or sufficient.

The strongest possible test of the hypothesis that brood pouch contents are responsible for the differences in behavior observed in Experiment 1 would pit the brood pouch stimuli against all other changes in the female's reproductive and molt physiology and determine whether the male bases his mate guarding behavior on the brood pouch contents, or on these other factors normally correlated with changes in the brood pouch contents. The next experiment was designed to perform this test.

Female *G. lawrencianus* are seldom guarded immediately after sperm transfer when the brood pouch is full of large recently fertilized, yolk-filled eggs which, according

TABLE I

The average time in seconds to the first palpation response by the male; the average time from the first palpation response to the precopula position, and the percentage of pairs that were in the precopula mate guarding position when the three minute test session was terminated

	Juveniles present in brood pouch	No juveniles in brood pouch
Average time to first palpation response	51.3 s	52.2 s
Average time from palpation response to precopula	80.1 s	25.3 s
Percentage mate guarding at end of test	100%	100%

to criteria described by Steele and Steele (1969), are Stage A embryos. Only two percent of the precopula mate guarding pairs observed in various samples from this breeding culture involve females carrying Stage A embryos. According to the reasoning outlined above, it should be possible to transform these unattractive, unguarded females into attractive, guarded females by aborting their embryos. An aborted female presents the male with a brood pouch which signals a short investment time, while all other aspects of her reproductive and molt physiology signal a long investment time.

Materials and methods

All maintenance conditions and test conditions used in the present experiment were identical to those described in Experiment 1, with exceptions noted below. For the present experiment, 30 unguarded females carrying Stage A embryos in their brood pouch were sampled from the breeding culture. The embryos were flushed from the brood pouch of 15 of these females (without apparent damage to the animals) by spraying a stream of seawater from a small glass capillary tube into the pouch. Another 15 females were exposed to a similar treatment directed at the outside of the brood pouch so that the embryos were sprayed but not removed. One hour after flushing, each female was placed in a test container with a male and observed for three minutes using the same behavioral assay described earlier. The males used in these assays were obtained from precopula pairs randomly selected from the breeding culture, separated from their female partners, and maintained in isolation for one hour prior to the behavioral assay in which they participated. A third comparison group consisted of 15 untreated, "normal" male-female precopula pairs randomly selected from the culture, separated for one hour and observed for three minutes as they re-established the precopula mate guarding position.

Results

The results of these treatments are seen in Table II. The important comparison is between the previously unguarded females carrying Stage A embryos and unguarded females at the same stage of the reproductive cycle with their embryos aborted. It is evident that a female with aborted embryos is more likely to be guarded by a male (53%) than a female at the same stage of the reproductive cycle with the same embryos

TABLE II

Average time in seconds to the first palpation response made by males; average time from palpation to the precopula position; and percentage of pairs in mate guarding position at the termination of the three minute test session

	Isolated females with embryos aborted (n = 15)	Isolated females with embryos in pouch (n = 15)	Precopula females with empty pouch (n = 15)
Average time to first palpation	30 s	35.6 s	41 s
Average time from palpation to precopula	83.3 s*	145.7 s	55.3 s
Percentage mate guarding	53%	6%	80%

* If only those males which successfully re-established precopula pairing with these females are considered, the average time from palpation to precopula was 24.6 seconds.

in her brood pouch (6%). However, aborting the embryos does not make the female as attractive as a "normal" guarded female with an empty brood pouch (80% mate guarding) ($\chi^2 = 16.55$, $P < .001$).

The second measure of interest in Table II is the time taken to make the precopulatory mate guarding decision once the male starts to palpate the female. The data reveal that the time taken from the first palpation response to the final precopulatory mate guarding response is significantly shorter for females with empty brood pouches, whether the embryos were aborted or vacated the pouch normally ($F = 8.8$, $P < .01$). It should be noted with reference to this decision time measure that the pairs which failed to achieve precopulatory mate guarding during the test period were assigned a decision time based on the interval between their first palpation response and the termination of the 3 min test session. In fact, the positive effects of the abortion procedure on decision time is even more impressive when one considers only those pairs in which precopulatory mate guarding was successfully established during the test session. The eight aborted females (53%) that were successfully guarded by males required an average decision time of only 24.4 seconds. This decision time was slightly faster than the average time required by the 12 (80%) successfully guarded "normal" females with empty brood pouches (33.7 seconds). Apparently, if the male decides to guard a female with an empty brood pouch, the decision time is short and approximately the same in response to aborted and normally empty brood pouches.

Finally, it should be noted that the amount of time required for the male to make the first palpation response did not differ in the three different groups of females ($F = <1$). Once again, brood pouch contents do not appear to have an effect on the attractiveness of the female upon initial contact.

DISCUSSION

The evidence from these two experiments demonstrates that the contents of the female brood pouch influences the male's mate guarding decision. This appears to be an adaptive reproductive strategy. The male amphipod's decision to guard a female with an empty brood pouch minimizes time and energy expended on the female and/or a previous male's offspring, and also assures the guarding male's paternity. To the extent that the brood pouch contains juveniles (or recently fertilized eggs), the decision to mate guard represents a more substantial investment of time and energy for the same gain (Parker, 1974).

The fact that a female with an aborted brood pouch is not quite as attractive as a "normal" female with an empty brood pouch (Exp. 2) suggests that factors in addition to the brood pouch contents are also contributing to the final precopula decision. One possibility is that the female actively participates in the mate guarding interaction. Females at different stages of their reproductive cycle may be more or less cooperative with the male, and this possibility needs to be examined with assays that are sensitive to changes in both male and female behavior during the mate guarding decision process.

Finally, these data do not exclude the possibility that a pheromone is involved in the precopulatory mate guarding of *G. lawrencianus*. They do, however, suggest that the communication process is more complex than previously considered. My observations of these animals, and descriptions of other species, suggest that there are two critical points in the mate guarding decision sequence: at initial contact the male either ignores the female or palpates her; and after palpation, the male either rejects the female or places her in the precopula position. The present data indicate that a different set of stimuli mediate these two different components of the mate guarding behavior.

Initial contact decisions may be based on a water borne or contact pheromone (*cf.* Hartnoll and Smith, 1980; Borowsky, 1984) and the subsequent precopula decision may be based on tactile, visual, and/or chemical information about the contents of the brood pouch. Techniques are currently being developed to systematically insert and extract various brood pouch contents to discover the stimulus dimension(s) which control the precopula decision.

ACKNOWLEDGMENTS

I thank Dr. Roger Croll for technical assistance and critical comments on the manuscript, and Dr. Roger Doyle for assistance in obtaining animals for the breeding culture. Research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada awarded to P. J. Dunham.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY: NORTHEASTERN REGIONAL DEVELOPMENTAL BIOLOGY CONFERENCE

FEBRUARY 21–23, 1986

The conference was organized by Susan G. Ernst, Tufts University, Medford, Massachusetts. Financial support was provided by the National Science Foundation and the Society of Developmental Biology. Best Student Presentation Award was sponsored by the American Society of Zoologists.

KEYNOTE ADDRESS

Molecular studies on biological rhythms in Drosophila melanogaster. MICHAEL ROSBASH, NADJA ABOVICH, HILDUR V. COLOT, JOHN EWER, JEFFREY HALL, AGNES C. JACQUIER, ANTHONY JAMES, GABRIELE PETERSEN, PRANHITHA REDDY, AND QIANG YU (Department of Biology, Brandeis University, Waltham, MA 02254).

The work presented concerns the function and expression of the *period (per)* gene of *Drosophila melanogaster.* This gene is intimately associated with the generation and/or maintenance of biological rhythms in this species, as mutations at the locus abolish or alter the nature of several biological rhythms. These include circadian rhythms and the short-term rhythm of the male love song. Germ-line transformation experiments indicate that *per*$^+$ biological activity can be localized to *ca.* a 6 kb region. This region contains the original *per*^{0} (arrhythmic) mutation and the *per*^{s} (short period) mutation and coincides with the coding region of a 4.5 kb transcript, the best candidate for the *per* gene mRNA. This mRNA is synthesized in mid-embryogenesis (*ca.* 8–18 h) and *in situ* hybridization experiments indicate that its expression is limited to the embryonic nervous system. It is resynthesized in late pupae and adults, where it is significantly enriched in heads. The sequence and structure of the *per* gene will be presented as well as the derived protein sequence. The most striking feature of the protein sequence is 20 consecutive pairs of Gly-Thr residues. A data base search detects homology to a mammalian chondroitin sulfate proteoglycan gene which contains *ca.* 25 consecutive pairs of Gly-Ser residues. By cloning an adjacent piece of coding DNA in an expression vector, generating an antibody reagent, and probing fly protein preparations, we have shown that the *per* gene does indeed code for a proteoglycan. The relevance of this finding to biological rhythms was discussed.

THE CYTOSKELETON AND EARLY EMBRYONIC PATTERNS

Ultrastructural identification of intermediate-sized filaments in Dictyostelium. S. T. KOURY AND B. S. ECKERT (Department of Anatomical Sciences, SUNY Buffalo School of Medicine, Buffalo, NY 14214).

We have examined heavy meromysin (HMM)-treated glycerinated cytoskeletons of the various stages of the *Dictyostelium* lifecycle (vegetative and aggregation stage amoebae, migrating and culminating pseudoplasmodia, mature stalk cells). Cytoskeletons prepared in such a manner exhibit actin filaments decorated with the characteristic arrowhead pattern and a second class of filaments of approximately 9 nm in diameter that do not react with HMM. The undecorated filaments are thus in the size range of intermediate filaments (IF) of other organisms. IF are rare in amoebae grown under axenic conditions, but become more common when amoebae are plated on agar and allowed to proceed through their lifecycle. The IF are often associated with bundles of decorated actin filaments in migrating pseudoplasmodia and appear to be found preferentially in basal cells of culminating pseudoplasmodia and stalks. The locations of IF in *Dictyostelium* are consistent with the hypothesized organizational and/or structural functions for IF in higher organisms and their discovery in *Dictyostelium* may provide a model system to study the function of IF during development.

A Microtubule-activated ATPase from sea urchin eggs. C. A. Collins and R. B. Vallee (Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545).

We have been analyzing ATPase activities from sea urchin egg cytosol with the aim of describing enzymes involved in microtubule-based motility. We have found that total ATPase activity in unfertilized egg cytosolic extracts is increased 30–50% upon assembly of endogenous tubulin with taxol, or upon addition of taxol-stabilized pure brain tubulin microtubules (MTs). The increased ATPase activity is recovered in a pellet after sedimentation of the MTs, and is, therefore, a MAP. The copurification of the ATPase with MTs is not affected by high ATP concentrations. The microtubule-activated ATPase can be distinguished from other cytoplasmic ATPases based on substrate specificity, divalent metal requirements, and response to pharmacological agents. The enzyme has been partially purified on sucrose density gradients, and migrates as a distinct peak at 10 S. Since MT assembly in the sea urchin egg occurs only after fertilization, a microtubule-activated ATPase may be expected to play a role in the regulation of early embryonic events such as assembly and function of MTs in the mitotic spindle.

Characterization of a surface contraction in Xenopus *eggs and oocytes.* Kathy Christensen and Robert W. Merriam (S.U.N.Y. at Stony Brook, Stony Brook, NY 11794).

Isometric contractions in the pigmented surface of *Xenopus* eggs are induced by adding calcium ions and ATP or GTP to an EGTA-containing buffer into which split eggs are placed. Half-cells extracted for 20 minutes in buffer fail to contract when calcium ions and nucleotide are then added back. Contractions can be regenerated if half-cells are extracted in the presence of myosin purified from *Xenopus* eggs or rabbit skeletal muscle. Neither egg- nor oocyte-soluble factors regenerate contractions in extracted oocytes. Myosin's ability to regenerate contractions in extracted half-cells is blocked by NEM-modified HMM from rabbit skeletal muscle, but unaffected by the calmodulin inhibitors TFP (trifluoperazine) and R24571 (calmidazolium), suggesting that the regulatory mechanism for the contractile activity studied with this assay system may not require calmodulin as a controlling factor.

Correlative vital fluorescence and immunofluorescence microscopy of organelles and the cytoskeleton in the mouse blastocyst. T. Ducibella, B. E. Batten, and D. F. Albertini (Departments of Anatomy and Obstetrics and Gynecology, Tufts University, Boston, MA 02159).

The objective of this study was to determine whether specific cellular compartments reorganized during the morula to blastocyst transition. Using acridine orange to stain acidic organelles and rhodamine 123 to stain mitochondria, we found that (1) acidic organelles change from a random to a perinuclear organization during the formation of the blastocoel, and (2) mitochondria are initially confined to the cell cortex in morulae and become concentrated around the nucleus in differentiated trophectoderm. Using rhodamine phalloidin, it was found that f-actin remains cortically associated during the morula to blastocyst transition. Immunofluorescence microscopy using rabbit anti-tubulin antibodies showed that cytoplasmic microtubules are basolaterally distributed in both morula and blastocysts; however, a perinuclear ring of microtubules characterized differentiated trophectoderm.

Contact induces coordinate redistribution of spectrin, myosin, and actin in mouse blastomeres. J. S. Sobel, J. M. Venuti, and E. G. Goldstein (Department of Anatomical Sciences, S.U.N.Y. at Buffalo, Buffalo, NY 14214).

Mouse blastomeres are characterized by a cortical layer of spectrin, myosin, and actin. When cultured on lectin-coated coverslips, which serve as a model for compaction, the proteins exhibit coordinate redistribution. Immunoreactive spectrin and myosin, together with rhodamine-phalloidin staining for F-actin, are lost from the contact region and the three proteins become concentrated in a ring around the contacted region. We also tested the ability of embryonic spectrin to bind to calmodulin using the ^{125}I-calmodulin-blot-overlay technique. Mouse brain and blastocysts both showed ^{125}I-calmodulin binding to a 240 kd polypeptide in the presence of m*M* CaCl$_2$ and dissociation of calmodulin in calcium-free buffer. We propose that contact modulation of the cortical cytoskeleton of the blastomere may be regulated, at least in part, via the influence of a Ca^{2+}-calmodulin on spectrin and its interaction with other proteins.

Induction of 8-cell blastomere polarization by embryonal carcinoma cells. R. R. ADLER AND C. A. ZIOMEK (Worcester Foundation, Shrewsbury, MA 01545).

The individual blastomeres of the preimplantation mouse embryo become polarized during the 8-cell stage. Microvilli become restricted to the free surface and this region of membrane shows increased labeling with FITC-Con A and TNBS. Previous studies have shown that this polarity develops in response to asymmetric cell-cell contact with stage-specific induction-competent blastomeres. Furthermore, the ability of cells to induce 8-cell blastomere polarization is lost with cellular differentiation. We have investigated the ability of embryonal carcinoma (EC) cells to induce polarization. Newly formed, nonpolar 8-cell stage blastomeres were isolated and aggregated with individual EC cells or cell clusters and cultured 8 to 10 h. We observed that cells of the pleuripotent cell line F9 would induce polarization in 80% of the aggregates. However, if the F9 cells were stimulated to differentiate by culturing them in 10^7 *M* retinoic acid the inducing ability of the EC cells decreased significantly. The nullipotent cell line Nulli-SCC1 induced polarization in only 30% of the aggregates while the more differentiated P19 cell line showed only 5% induction. The ability of isolated cells from adult mouse tissue to induce 8-cell blastomere polarization has also been investigated. We found that freshly isolated mouse lymphocytes induced polarization in only 11% of the aggregates, while STO cells, a cultured line of mouse fibroblasts, showed only 17% induction. These results support the hypothesis that the ability of cells to induce polarization is lost with cellular differentiation.

EPIGENETIC FACTORS OF NEURAL DEVELOPMENT

Cellular and molecular mechanisms of neuron-glial interactions in vitro. MARY E. HATTEN (New York University School of Medicine, New York, NY 10016).

With cellular antigen markers, cerebellar neuron-glial interactions can be studied in an *in vitro* model system. Time-lapse video microscopy of living cells correlated with electron microscopy reveals that migrating granule neurons extend a thickened leading process along and form punctate contacts with the astroglial arm. Cells from the mutant mouse weaver fail to form migration appositions, even when positioned on wild-type astroglia, suggesting that the granule neuron directs migration along the glial arm. Using this culture system as a functional assay, a poly-specific antiserum reversibly blocks neuron-glial interactions. This antiserum binds to a cell surface glycoprotein of molecular weight 92 kd.

In the absence of neurons, astroglia fail to differentiate and proliferate rapidly. Recombination of neurons with glia and experiments with purified neuronal membranes indicate that neurons regulate astroglial differentiation by contact-mediated events.

These studies underscore the importance of epigenetic, cellular interactions to both neuronal and astroglial development in the mammalian brain.

Protease involvement in neurite outgrowth. RANDALL N. PITTMAN (Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA 19104).

Rat sympathetic neurons grown in culture release a calcium-dependent metalloprotease(s) and a urokinase-like plasminogen activator from their distal processes and/or growth cones (Pittman. 1985. *Dev. Biol.* **110**: 91). An affinity-purified antiserum directed against urokinase (UK) as well as inhibitors of UK increase neurite outgrowth on laminin but not on Type I collagen. This effect does not appear to result from blocking the activation of a zymogen (*e.g.*, plasminogen), or the proteolysis of an ECM or membrane constituent by UK. A protein on the surface of neurons binds UK as well as a component of the ECM and therefore may be involved in this UK-dependent neurite outgrowth.

EXTRACELLULAR MATRIX IN DEVELOPMENT

Hyaluronate-cell interactions in embryonic development. BRYAN P. TOOLE (Department of Anatomy and Cellular Biology, Tufts Medical School, Boston, MA 02111).

Hyaluronate is a major component of the extracellular matrix which surrounds proliferating and migrating cells at early stages of morphogenesis of embryonic tissues and organs. Using various cell lines *in vitro* we have demonstrated two distinct modes of interaction of hyaluronate with the cell surface. The first is a stable interaction with the plasma membrane which is typical of cells with large pericellular coats of hyaluronate but is of unknown mechanism. The second involves specific, non-covalent, high affinity interaction of the polysaccharide chain with multiple receptors in the plasma membrane. A sudden transition from the former to the latter type of interaction occurs at the stage of mesodermal condensation in the chick embryo limb bud. During subsequent chondrogenesis the hyaluronate receptors are retained and mediate assembly of the chondrocyte pericellular matrix whereas during myogenesis the receptors are lost. It is proposed that these changes are involved in regulation of cell interactions leading to differentiation.

Extracellular matrix and the effect of nerves during limb regeneration. S. I. MUNAIM AND A. L. MESCHER (Medical Sciences Program, Indiana University, Bloomington, IN 47405).

The extracellular matrix (ECM) of the distal tissues in a newt limb stump is completely remodelled in the 2–3 week period following amputation resulting in an environment favorable for cell migration and proliferation. This work was done to test the view that one way by which nerves can affect blastema cell proliferation is by influencing the ECM metabolism. Using biochemical techniques, hyaluronic acid (HA) was found to be the major glycosaminoglycan (GAG) produced during the nerve-dependent phase of dedifferentiation and chondroitin sulfate during differentiation. Histochemical staining with Stains-all and autoradiographical studies confirmed the biochemical data and showed the uniform distribution of these GAGs in the distal tissues except for a heavier localization in the periosteum. Denervation caused a 50% reduction in GAG synthesis. Newt dorsal root ganglia and fibroblast growth factor each caused GAG synthesis to be doubled in cultured 7-day regenerates, the latter effect being primarily on HA production. These data indicate that nerves affect the synthesis and accumulation of ECM components and this neurotrophic action may be important for the processes of cell migration and growth in the early regenerate.

Tumor cell-fibroblast interactions and extracellular matrix modulation. C. BISWAS (Department of Anatomy and Cellular Biology, Tufts Medical School, Boston, MA 02111).

Cell interactions and changes in extracellular matrix composition play fundamental roles in normal embryonic development. Tumor cells and embryonic cells share many common characteristics in terms of cell behavior and biochemical properties. We have investigated two parameters of extracellular matrix modulation which are associated with tissue development and remodelling as well as with tumor invasion and metastasis. These are (1) collagenase, which participates in removal of structural barriers to cellular invasion and (2) hyaluronate, which is a major component of extracellular matrices surrounding migrating and proliferating cells in the embryo. In both cases we have shown that tumor cells interact with fibroblasts to cause increased production of these two components in the fibroblasts and that these effects are mediated via factors present within the tumor cell plasma membrane. Our conclusion is that the interactions between tumor cells and fibroblasts give rise to alterations in the host extracellular matrix which facilitate tumor cell invasion through this matrix.

The role of extracellular matrix in the outgrowth of parietal endoderm from teratocarcinoma embryoid bodies. L. B. GRABEL, J. E., CASANOVA, AND T. D. WATTS (Department of Biology, Wesleyan University, Middletown, CT 06457).

Teratocarcinoma embryoid bodies consist of an inner stem cell core surrounded by an outer endoderm layer and provide an excellent *in vitro* system for studying certain events during mouse embryogenesis. We report here that the selective outgrowth of parietal endoderm cells can be studied by plating these embryoid bodies on appropriate substrates. Embryoid bodies that do not initially contain parietal endoderm appear to generate parietal endoderm when plated on type I collagen substrates, suggesting the inductive potential of matrix interactions. We have also evaluated a number of extracellular matrix glycoproteins for their ability to promote endoderm outgrowth and have determined that fibronectin is substantially more active than laminin or type IV collagen. The same hierarchy exists for embryoid body attachment. These results have *in vivo* relevance since fibronectin is observed on the inner trophectoderm surface, the site of parietal endoderm migration in the embryo.

GENE EXPRESSION DURING DEVELOPMENT

Sequences and splices governing CNS expression of the Drosophila *dopa decarboxylase gene* Ddc. S. J. BRAY, S. B. SCHOLNICK[1], B. A. MORGAN, C. J. BEALL, AND J. HIRSH (Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115, and [1]Department of Biological Sciences, Carnegie-Mellon University).

We have identified cis-acting sequences and an alternate splice which are responsible for the CNS expression of the *Drosophila melanogaster* dopa decarboxylase gene *Ddc*. Previous studies have shown that essential regulatory elements cannot be farther than 208 bp upstream of the RNA startpoint. We have identified several repetitive sequences in this 5′ flanking region which are conserved through evolution between two distantly related fly species. The functions of two of these sequences were discerned by deletion analysis, assaying expression *in vivo* following germline integration via P element vectors. One of these elements is necessary for quantitatively normal expression throughout development, whereas the other has selective effects on central nervous system expression.

We have characterized the *Ddc* transcript in the central nervous system. The CNS transcript uses an alternative splice which has the potential to encode an altered protein. Studies are in progress to assay the physiological role of this transcript.

Determination of the muscle cell lineage in ascidian embryos. THOMAS H. MEEDEL, ROBERT J. CROWTHER, AND J. R. WHITTAKER (Marine Biological Laboratory, Woods Hole, MA 02543).

The results of ascidian cell lineage studies first reported by Conklin and later by Ortolani indicated that all larval muscle cells originate from progeny of the posterior-vegetal blastomeres (B4.1 pair) of the 8-cell embryo. When B4.1 blastomeres from *Ciona intestinalis* embryos are isolated and the quarter-embryos raised to "maturity" some of their descendents differentiate muscle; progeny of the other three isolated pairs of blastomeres do not develop muscle. Such a finding strongly supports the belief that cells of the B4.1 lineage are the sole precursors of muscle. This view has recently been challenged by the results of cell marking experiments using horseradish peroxidase: some muscle cells appear to originate from each of the anterior-vegetal (A4.1) and posterior-animal (b4.2) lineages. Based on biochemical, histochemical, and ultrastructural analyses of muscle traits in isolated partial embryos we confirm that the A4.1 blastomeres contain a lineage for muscle cells. Differences in the timing of expression and their need to associate with other cell lineages in order to differentiate suggest the determination of fate in A4.1 derived muscle depends on mechanisms different from those operating in the B4.1 lineage.

Isolation and expression of the zerknullt *locus of* Drosophila.* TIM HOEY, HELEN DOYLE, KATHERINE HARDING, AND MICHAEL LEVINE (Columbia University, New York, NY 10027).

Elaboration of postional identity along the anterior-posterior and dorsal-ventral embryonic body axes involves early zygotic gene functions that are expressed in response to maternal cues present in the unfertilized egg. Zygotic genes that are required for the specification of positional identity along the anterior-posterior body axis have been described in detail. Mutations in many of these genes disrupt the characteristic segmentation pattern of the embryo. The *zerknullt* (*zen*) locus appears to be one of the zygotic genes required for proper tissue differentiation along the dorsal/ventral axis. Advanced-stage *zen* embryos show a complex syndrome of defects including a severe twisting or buckling along the anterior-posterior axis, and a failure of head involution. Many of the defects seen in older *zen* embryos appear to arise as secondary consequences of a primary lesion that occurs during early stages of development. In particular, there is a failure of the germ band to fully elongate (during ~5–6 hours post-fertilization). It is possible that incomplete elongation of the germ band results from the absence of several of the dorsal-most embryonic tissues, including the amnioserosa. Despite the distortions of the germ band, a normal number of body segments are formed.

The molecular cloning of *zen* has permitted corroboration of previous proposals regarding *zen* function during early embryonic development and further establishes a role for *zen* in the differentiation of tissues that derive from the dorsal ecotderm. Since *zen* contains a well-conserved homeo box sequence, it appears that differentiation of the anterior-posterior and dorsal-ventral patterns involves similar molecular mechanisms.

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* Best student platform presentation.

Genes involved in limb formation in the mouse. R. WOYCHIK, R. MAAS, R. ZELLER, T. STEWART, P. D'EUSTACHIO, AND P. LEDER (Department of Genetics, Harvard Medical School, Boston MA 02115).

In the preparation of transgenic mice, several hundred DNA fragments containing the gene of interest are injected directly in the male pronucleus of the fertilized egg. Several of these injected DNA fragments typically insert into the host genome, usually at a single site, and become a stably heritable genetic trait. In one line of transgenic mice prepared in our laboratory, insertion of the exogenously added DNA fragments appears to have resulted in a recessive mutation that gives rise to a limb deformity in homozygous animals. To test the simple hypothesis that insertion of the exogenously added DNA fragments disrupted the function of a gene that normally participates in regulating limb morphogenesis, several kilobases of the mouse genomic sequence flanking the insertion site were cloned utilizing a portion of the injected DNA fragments as a molecular "tag." We are currently characterizing several regions of sequence which are highly conserved through evolution and may represent the exons of the interrupted gene in our transgenic mouse. DNA fragments containing these putative exon regions are currently being utilized in an attempt to identify the mRNA expressed from this region of the mouse genome. Interestingly, complementation analysis has revealed that this mutation is allelic with two other independently characterized mutations that give rise to the same phenotype.

Genes transcribed early in Xenopus *development.* RICHARD P. HARVEY, PAUL A. KRIEG, CLIFFORD J. TABIN, AND DOUGLAS A. MELTON (Harvard University, Cambridge, MA 02138).

One of the major problems in vertebrate embryology is how to identify genes which regulate development and/or pattern. Here, we describe two *Xenopus* genes, GS17 and Xhox-1A, which may function in developmental decision-making. GS17 was selected from among genes which are expressed precisely at the time when the embryonic genome becomes transcriptionally active. Its transcripts are present in only a narrow period of developmental time, implying a role in the earliest embryonic events. Xhox-1A was selected because it contains a conserved homeobox sequence. It is transcribed both maternally and after mid-gastrulation.

Our experiments are directed towards understanding both the function and regulation of these genes. These analyses will require an ability to manipulate the expression of genes in the developing embryo. Transcription from cloned GS17 genes introduced into developing embryos by microinjection is correctly regulated. This includes both on and off transcription signals. Similarly, synthetic Xhox-1A mRNAs can be translated in *Xenopus* oocytes and the homeobox protein produced localizes to the nucleus. We will discuss ways of using exogenous expression to address questions of function and regulation.

Genes affecting sex determination and dosage compensation in the nematode Caenorhabditis elegans. BARBARA J. MEYER (Massachusetts Institute of Technology, Cambridge, MA 02139).

The ratio of X chromosomes to sets of autosomes determines whether a nematode develops as a self-fertile hermaphrodite (2X:2A) or as a male (1X:2A). Moreover, because sexual differentiation is determined by the chromosome composition, the nematode has imposed a further mechanism of dosage compensation to ensure that expression of genes on the X chromosome is kept equal in the different sexes. One area of research in our laboratory, in addition to demonstrating the existence of dosage compensation, has been to gain an understanding of how *C. elegans* assesses the ratio of X chromosomes to autosomes and how this measurement controls the expression of sex-determining genes and dosage-compensating genes downstream in the pathway. The data we have accumulated on the gene *egl-16* are consistent with a model in which the wild-type *egl-16* activity is involved in transmitting information about the X/A ratio to both the sex determination and dosage compensation pathways. A mutation in *egl-16* results in under-assessment of the X/A ratio in an XX animal, shifting sex determination and dosage compensation mechanisms toward their XO modes. The manifestations of a mutation in *egl-16* which lead to this model are that (1) the mutation can promote sexual transformation of XX animals toward males and enhance the transformation phenotypes of other weakly masculinizing mutations and (2) the mutation disrupts dosage compensation in XX animals resulting in overexpression of the X chromosome in hermaphrodites but not males.

CONTRIBUTED PAPERS

Actin-like protein as the major component of insect oocyte cortical cytoskeleton. S. J. BERRY AND B. JARNOT (Department of Biology, Wesleyan University, Middletown, CT 06457).

The cortex of insects is a strategically important region because it is the major site of cell formation for the embryo. We have examined the cortex of the eggs of several species of giant moths, and have uncovered a substantial cytoskeletal system confined to this region. The major protein component of the cortical cytoskeleton is actin. The actin is not synthesized in the egg, but appears to be transported from the fatbody via the blood, just as vitellogenins are. We are convinced that a 42 kd protein formerly thought to be yolk protein is, in fact, actin. Actin is demonstrated in blood, fatbody, and cortical cytoskeleton by a number of techniques including antibodies, fluorescent staining, affinity chromotography, and electrophoresis.

Centrosome doubling does not require nuclear DNA synthesis. G. SLUDER AND K. LEWIS (Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545).

The importance of nuclear DNA synthesis in the reproduction, or doubling, of centrosomes prior to each mitosis has been uncertain. We systematically examined the long-term effects of alphidicolin on centrosome reproduction in sea urchin eggs. This agent specifically inactivates the α DNA polymerase thereby inhibiting nuclear DNA synthesis. Eggs of three species of sea urchins were treated with 5 or 10 μg/ml aphidicolin starting a few minutes after fertilization. We find that 5 μg/ml reduces the incorporation of ^{3}H thymidine into DNA by greater than 90%; 10 μg/ml inhibits slightly more. We found that 5 or 10 μg/ml aphidicolin slowed but did not prevent at least the first three cycles of centrosome reproduction, at which point we terminated our detailed observations. In a variable percentage of eggs the nucleus never broke down and became grossly stretched out between the asters. At later cycles, the nucleus was not engaged by all asters. In the remaining eggs, the nucleus broke down but never reformed. Fluorescent labeling of chromatin revealed that proper chromosome condensation never occurred. The chromatin was a tangled mass stretched out like a thick thread between the asters. All three species of urchins showed the same response to aphidicolin.

The behavior of cyclin A protein during early clam development. K. I. SWENSON AND J. V. RUDERMAN (Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115).

The oocytes of the surf clam, *Spisula solidissima,* contain a pool of maternally stored mRNAs which are not utilized for protein synthesis until after the time of fertilization when they become rapidly loaded onto polysomes. We are interested in the functions of the proteins encoded by these mRNAs in the rapidly dividing embryo. Two of the proteins, cyclins A and B, which are strongly activated after fertilization are periodically destroyed as a function of the cell cycle. By subcloning a portion of a cDNA clone specific for cyclin A into a procaryotic expression vector, we were able to make and isolate a large amount of a bacterial/cyclin A fusion protein. This fusion protein was used to immunize rabbits resulting in antisera which recognized not only the bacterial fusion protein but the clam cyclin A protein as well. We used these antisera to probe immunoblots containing total clam proteins obtained at different times following fertilization. This study showed that cyclin A underwent a periodic accumulation and degradation not only during mitosis, but meiosis as well. Furthermore, the time of degradation appeared to be concomitant with the time of the metaphase/anaphase transition of the cell cycle. We suspect that cyclin A is an important protein involved in the mechanisms which drive cell division in the early embryo. Preliminary immunofluorescence studies indicated that cyclin A was associated with the other cellular membrane.

Ontogeny of avian activity rhythms. C. J. FISHER[1], AND P. J. DECOURSEY[2] ([1]Vassar College, Poughkeepsie, NY 12601 and [2]University of South Carolina, Columbia, SC 29028).

Shell-less culture of chick embryos facilitated direct observation of developing rhythmicity. Muscle activity was studied in white leghorn chick embryos from the start of culture on day 3 of development to day 19, the limit of survival. Rates of amniotic (smooth) muscle and skeletal muscle contraction under constant lighting and temperature conditions were measured. Amniotic contraction commenced on day 4, increased through day 10, and then dropped off sharply. Skeletal muscle contractions, expressed as % time active, began about day 7, correlated with rapid limb growth, and continued as long as embryos survived. Neither individual data nor pooled means for data through day 12 of development suggested circadian rhythmicity. From days 13–17 small, short-term fluctuations in rate of skeletal muscle contraction were seen, which increased gradually in amplitude and period. On days 13–19, changes in activity rate from about 9% to 69% at approximately 12-hour intervals suggested incipient rhythms which may lengthen into circadian rhythms. Supported by NSF PCM-8402332 and NSF PCM-8402104.

Juvenile hormone-like compounds in Crustacea. H. LAUFER[1], D. W. BORST[1], F. B. BAKER[2], AND D. A. SCHOOLEY[2] ([1]The University of Connecticut, Storrs, CT 06268, and [2]the Zoecon Corporation, Palo Alto, CA 94305).

Juvenile hormone (JH) is known to play important roles in insect development and reproduction. There may be JH-like compounds in crustacea. One possible source of such compounds is the mandibular organ (MO), a tissue that has been shown by others to affect reproduction in the spider crab, *Libinia emarginata*.

To test this hypothesis, we incubated MOs from several crustacea, including *Libinia, Callinectes sapidus*, and *Homarus americanus* in Pantin's or lobster saline for 2 hours supplemented with methyl-labeled methionine. MOs secreted radiolabeled methyl farnesoate (MF) into the culture medium as revealed by HPLC and GC/MS. The synthetic rate for MOs from *Libinia* ranged from 3 to 38 ng/gland/h. MF was also detected in hemolymph samples from *Libinia* with circulating levels of 10 to 50 ng/ml.

Our data demonstrate that MF is a tissue-specific and a major secretory product of the crustacean MO. Since MF has JH activity in insects, and its concentration is correlated with egg production in reproductive females, this compound may be a crustacean JH.

Identification of a binding protein for retina cognin in chick embryonic retina cell membranes. N. M. TROCCOLI AND R. E. HAUSMAN (Biological Science Center, Boston University, Boston, MA 02215).

Retina cognin (R-cognin) is an intrinsic membrane glycoprotein of vertebrate retinal cells which supports tissue-specific cell adhesion and mediates cell-type specific associations during development. The ability of anti-cognin antibody to inhibit these interactions suggests that R-cognin acts directly to link the cell membranes. Does R-cognin do so by binding to another R-cognin in the apposing membrane (homophilic interaction) or does it bind a different protein (heterophilic interaction)? To answer this, we constructed an affinity column with cognin-enriched membrane proteins conjugated to the column matrix. Detergent-extracted retina membrane proteins were applied to the affinity column and those which bound specifically eluted and visualized by electrophoresis. A protein of 64 kd was found. To confirm that the 64 kd protein bound to R-cognin, we examined the cognin-dependent aggregation of retina membrane vesicles. When aggregation was preformed in the presence of a photo-activated crosslinker, a 114 kd cognin-containing complex was formed. This can be resolved into a 50 kd cognin and 64 kd binding protein under reducing conditions. Additional techniques are being pursued to identify the protein.

POSTER ABSTRACTS

Retinoic acid induces fibronectin fiber formation in F-9 teratocarcinoma stem cells. S. C. DAHL, AND L. B. GRABEL (Department of Biology, Wesleyan University, Middletown, CT 06457).

F-9 teratocarcinoma cells differentiate into parietal endoderm when monolayer cultures are treated with retinoic acid. This change in phenotype is accompanied by an overall increase in the synthesis and accumulation of extracellular matrix. We have used indirect immunofluorescence to monitor the accumulation of fibronectin during the differentiation process and have documented a dramatic difference in the deposition of this matrix component in untreated and treated F-9 cultures. Untreated cells deposit fibronectin in a punctate, membrane associated pattern, whereas treated cells form fibrous networks which transcend cell boundaries. One and 2-D gels of conditioned medium suggest that the two cell types secrete similar, if not identical, fibronectins. Furthermore, preliminary results indicate that both cell types express the 140 kd fibronectin receptor on their surface. Thus, our data suggest the possibility of another receptor or factor necessary for fiber formation.

Preliminary observations on cortical granule composition in eggs of Brachydanio. [illegible] N. HART, AND J. WOLENSKI (Department of Biological Sciences, Rutgers University, New Brunswick, NJ 08903).

Studies have been initiated on the composition of cortical granules in *Brachydanio* (teleost) eggs to examine the potential role of cortical granule exocytosis in the development of the vertebrate egg. Two approaches were used: (1) application of selected cytochemical tests to sections of paraplast-embedded unfertilized eggs; and (2) SDS-PAGE of cortical granule exudate. Cytochemically, cortical granule contents showed positive reactions with toluidine blue, thionin, alcian blue 8GS, astra blue, and periodic acid-Schiff reagent. This indicated the presence of glycoproteins with the carbohydrate moiety consisting of simple carboxylated and sulfated acid mucopolysaccharides. A method was developed to collect cortical granule exudate from 10-minute activated eggs. Supernatant (with chorions) from 50–75 eggs was analyzed by SDS-PAGE using a 4% stacking gel with a 10% running gel. Four faintly staining proteins were detected with Coomassie Blue R-250 and 12 proteins with silver stain (Biorad). Molecular weight standards ranged from 29K to 120K. Since chorions appeared to resist SDS treatment during sample preparation, the resolved proteins are probably constituents of the cortical granules.

Characterization of an unusual, evolutionarily conserved sea urchin H2A histone gene. S. G. ERNST, C. A. BRENNER, S. FRANCIS, H. MILLER, AND R. MCISAAC (Department of Biology, Tufts University, Medford, MA 02155).

In sea urchins, members of the histone multigene family have a coordinated pattern of differential expression. A *S. purpuratus* clone has been isolated from a gastrula cDNA library. The clone was sequenced and a computer search for the identity of the clone revealed striking homology with a variant histone gene in chickens, H2A.F. The sequence shows 98% amino acid conservation and 73% nucleotide homology with the chicken H2A.F gene. The sea urchin H2A.F mRNA is unusual in that it is about three times longer than a typical histone mRNA and it is found in the polyA$^+$ fraction from oligo dT cellulose. H2A.F mRNA is stored in the egg and detectable at the 16-cell, mesenchyme blastula, gastrula, and pluteus stages. Whole genome Southern blot analysis indicates that this sequence is present one to a few times in the sea urchin genome. The early histone genes are represented 400 times and the late histone sequences are found 5–12 times per genome. The H2A.F gene codes for the sea urchin H2A.Z histone protein.

The developing neuromuscular junction in neonatal rats treated with ACTH fragments: a comparison of scanning electron microscopy and light microscopy. R. E. FRISCHER AND F. L. STRAND (Biology Department, New York University, New York, NY 10003).

Neonatal rats were injected with ACTH 4–10 (10 μg/kg/day s.c.); ACTH 4–9 (Org 2766) (0.01 μg/kg/day s.c.) or saline from birth to two weeks of age. The development of the neuromuscular junctions (NMJs) in the extensor digitorum longus muscle of these 14-day-old rats was morphologically compared using both light and scanning electron microscopy (SEM). The developing NMJs visualized through SEM reveal structural aspects, as well as a three-dimensional topography of the postjunctional folds that are extrusions of the endplate area rather than the invaginations as seen in mature animals. These results are contrasted with silver nerve stain and cholinesterase histochemistry revealing axonal branches converging into a single endplate per myofiber. The use of both techniques thus provide very different information about the developing NMJ.

Synthesis and accumulation of small cytoplasmic RNAs in sea urchins. J. M. LEBLANC AND A. A. INFANTE (Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06457).

The presence, subcellular localization, and synthesis of small RNAs in developing sea urchin embryos (*S. purpuratus*) were investigated. Cytoplasmic extracts were prepared from embryos labeled *in vivo* with [^{3}H]-uridine/adenosine at different stages of development. These extracts were separated into polysomal and free RNP (10-70S) fractions. PAGE analysis of RNA extracted from these fractions revealed many small RNAs in the free RNP fraction. Fluorographed gels indicated that some small RNAs are not synthesized until the late morula-early blastula stage. One of these labeled RNAs, which is prevalent at all developmental stages, migrates at 7S, and may be equivalent to the 7S RNA present in the mammalian signal-recognition-particle (SRP). To test this possibility, labeled embryos were homogenized in 0.5 *M* KCl to free SRP from any membrane association, and the free RNP fraction centrifuged in sucrose gradients. The 7S RNA sedimented at about 10S. Northern blots of total cellular RNA were probed with a human cDNA clone of the 7SL RNA of the SRP (a kind gift from E. Ullu) and showed that the band migrating at 7S does contain this small RNA. (Supported by NIH grant GM31605).

Morphological evidence of a guidance system present during Rana pipiens gastrulation. J. LEBLANC[1], M. YODER[2], AND I. BRICK[2] ([1]The College of Staten Island, CUNY, NY 10301 and [2]NYU, NY 10003).

Rana pipiens embryos from late blastula through late gastrula are being examined utilizing scanning and transmission electron microscopy. In *Rana*, as in other amphibians, the mechanisms involved in cell migrations, such as cell guidance factors, during gastrulation for the most part remain, unresolved.

One region being investigated by electron microscopy, as part of an ongoing *Rana* gastrulation study, is the area of the inner ectodermal surface anterior to the translocating fold, the fold being comprised of mesodermal and endodermal cells. The ectodermal area anterior to the migrating fold, for a distance varying from 3 to 5 cell lengths, exhibits long cell processes aligned along the posterior-anterior axis. Filopodia and lamellipodia extending from the leading-edge cells of the fold appear to be making or to have made contact with the processes of the ectodermal cells; the processes of the ectodermal cells may act as guidance factors in the migration of leading-edge cells *in vivo.*

Maternal effect mutations in the nematode Caenorhabditis elegans. D. J. LEVITAN, A. TELFER, AND D. T. STINCHCOMB (Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138).

We are identifying and characterizing maternal effect mutations that perturb early embryogenesis in *C. elegans.* To screen for such mutations we use strains that fail to lay eggs. In these strains larvae hatch within the hermaphrodite, causing a characteristic "bag of worms" phenotype. Mutants are easily identified as worms that accumulate unhatched embryos. Our screens are designed to pick up three classes of mutations: dominant and recessive mutations induced by EMS mutagenesis, and recessive mutations caused by the insertion of the transposable element Tc1. We have isolated variants in each of the three classes. Strains bearing a putative insertional mutation show striking abnormalities in the early lineage and the embryos fail to undergo morphogenesis and normal cell differentiation. We will continue to characterize these mutants both phenotypically and genetically with an eye toward elucidating their defects at the molecular level.

Identification and characterization of sea urchin transcription factors. P. E. NISSON, AND W. R. CRAIN (Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545).

The muscle, pSpG28, and cytoskeletal, pSpG17, actin genes from the sea urchin, *S. purpuratus* are differentially regulated at the level of transcription (Hickey, Boshar, and Crain, unpub.). Transcription of the cytoskeletal gene is first detected in 11 h embryos, whereas transcription of the muscle gene is not detected until the pluteus stage (72 h). These findings suggest that transcription regulatory factor(s) may be required for this differential gene expression. To test this hypothesis, we have initiated a study to identify and characterize transcription factors that regulate the expression of these two genes. The first objective has been to determine if, and with what accuracy, the two sea urchin genes are transcribed in *Xenopus laevis* oocytes. By northern blot analysis we have shown that both genes are transcribed in *Xenopus* oocytes after injection into the germinal vesicle. Mapping of the ends of the transcripts from these genes indicates that transcription of the sea urchin muscle gene is both initiated and terminated accurately in *Xenopus* oocytes. For the cytoskeletal gene only the 3′ end of the transcript has been mapped so far, and it shows accurate termination. We are now in a position to identify chromatin-associated factors which activate or repress transcription of these two actin genes by co-injection into *Xenopus* oocytes.

Protein carboxyl methylation in Xenopus laevis *oocytes: a mechanism for protein repair?* CLARE M. O'CONNOR (Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545).

A protein, methyltransferase, has been identified in *Xenopus laevis* oocytes which is similar in its physical properties, substrate specificity, and antigenic properties to an enzyme in mammalian tissues which [illegible] protein D-aspartyl and L-isoaspartyl residues. These unusual amino acids which act as substrate are [illegible] derived from spontaneous racemization and deamidation reactions which accompany the aging of proteins in cells, and it has been hypothesized that the function of this enzyme involves the repair or metabolism of its damaged protein substrates. The concentration of the methyltransferase in vitellogenic oocytes is approx. 3 μM, and the enzyme is distributed in both the germinal vesicle and cytoplasm of oocytes. We have identified numerous proteins in both the germinal vesicle and cytoplasm which are methylated following the injection of oocytes with S-adenosyl-[methyl-^{3}H]methionine. From kinetic analysis of methyl group incorporation, we have shown that protein carboxyl methylation is the major methylation pathway in *Xenopus* oocytes.

Free calcium pulses during early development of Ciona *eggs.* J. E. SPEKSNIJDER, D. W. CORSON, T. H. QIU, AND L. F. JAFFE (Marine Biological Laboratory, Woods Hole, MA 02543).

Ascidian eggs serve as interesting systems for the study of early development, since they show a classic example of ooplasmic segregation and then develop into tadpoles within a day. We have begun to explore the role of free cytosolic calcium during the early development of *Ciona* eggs by injecting them with the calcium-specific photoprotein, aequorin, and then observing their luminescence after adding sperm.

Such an aequorin-loaded *Ciona* egg regularly shows an enormous pulse of luminescence which starts 1 to 5 minutes after insemination. In 30 to 60 seconds, luminescence rises to a peak level which is on the order of 10,000-fold above instrumental background. Assuming a resting free calcium level of 0.1 μM and a square law dependence of light emission on free calcium, we estimate that free calcium rises more than 300-fold to a peak level of about 30 μM. The luminescence then gradually subsides, returning to the background level in about 5 minutes. These pulses resemble those known in medaka fish eggs as well as urchin eggs and presumably accompany egg activation and ooplasmic segregation.

Such eggs also regularly show a remarkable series of smaller and briefer pulses in the period between the end of the activation pulse and first cleavage. These postactivation pulses occur in two series—an early group of 3 to 5 pulses which start soon after the activation pulse subsides (and may accompany first polar body formation) and a late group of 8 to 12 pulses which start about 25 minutes after fertilization (and may accompany second polar body formation). However, no changes in luminescence are observed during first cleavage.

This first study utilized a simple photon counter and gives no spatial information; but we plan to re-examine these *Ciona* egg pulses with an imaging photon detector to determine just where free calcium rises in the egg.

Transcription of germ line specific DNA in Tetrahymena. SUSAN STEIN-GAVENS, JOHN M. WELLS, AND KATHLEEN M. KARRER (Brandeis University, Waltman, MA 02254).

In the ciliated protozoan *T. thermophila* 10–20% of the DNA sequences are micronucleus (germ line)-specific. They are eliminated from the developing macronucleus during sexual reproduction (conjugation). The function of germ line-specific DNA in *Tetrahymena* is unknown. Most of the micronucleus-specific sequences are members of repeated families. We cloned a 0.7 kb HindIII-ClaI fragment that is a member of such a family. Using this cloned sequence as a probe, we detected a 1.5 kb poly A^+ transcript on northern blots. The transcript was not seen in RNA prepared from vegetatively grown cells, but was detected in RNA from starved cells (a condition necessary to bring about conjugation). The abundance of the transcript decreased as the cells entered conjugation. The northern signal can be washed off under moderately stringent conditions, suggesting that a different member of the family is the template for the transcript. Five other members of this repeated family were isolated from a micronuclear genomic bank. When the new clones were hybridized to northern filters, they detected a transcript with the same size and inducibility as seen with the original clone, consistent with the hypothesis that a member of the micronucleus-specific family is transcribed. Supported by GM32989 from the National Institutes of Health.

Morphology of regenerated retinal axonal terminal arbors and growth cones in the goldfish tectum. C. A. O. STUERMER, M. BECKMANN, AND E. KALKO (Max-Planck-Institut für Entwicklungsbiologie, Spemannstr. 35, D-7400 Tübingen, FRG).

Regenerating goldfish retinal axons and terminal arbors were labeled by applying HRP to cut axon bundles in the retina at defined retinal positions at early, intermediate, and late regeneration stages.

At all stages, one group of axons was correctly routed and terminated at retino-topic sites. Another group was misrouted. These axons were unbranched where they coursed through the superficial fascicle layer. Upon entering the synaptic layer, however, they exhibited tortuous trajectories and innumerable filopodial processes. Or they extended two or more long branches, each studded with one or more growth cones, into various directions of the tectum.

At late regeneration stages, the axons had lost most of their branches, reached their retinotopic sites through abnormal routes, and deployed their terminal arbors correctly. Thus, a considerable number of regenerating axons appear to go through a phase of "exploratory growth" before they find their correct retinotopic termination area.

Nerve growth factor regulates both cAMP-dependent and cAMP-independent protein kinase activities. R. VAN BUSKIRK AND J. A. WAGNER (Department of Biological Chemistry, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA).

We have resolved five protein kinases present in PC12 cells and show that the activity of two of the five kinases are regulated by nerve growth factor (NGF) treatment. The first major kinase to elute using fast protein liquid chromatography (0.10 *M* NaCl) is Type I cAMP-dependent protein kinase. There is no change in activity of this kinase when cells are treated with NGF. The second activity to elute (0.20 *M* NaCl) may be a derivitive of the Type I cAMP-dependent protein kinase. This activity is cAMP-independent in extracts of untreated cells, but if cells are treated with NGF for at least two hours this activity is cAMP-dependent. The third and fourth kinase activities to elute (0.30 and 0.35 *M* NaCl) are two subtypes of the Type II cAMP-dependent protein kinase. The activities of these kinases are not altered when cells are treated with NGF. The fifth kinase activity (NRK for NGF-regulated kinase) elutes at 0.40 *M* NaCl and is not detectable in untreated extracts when histone type 2a is used as a substrate; however, when cells are treated with NGF for at least 2 hours, a kinase activity eluting at 0.40 *M* NaCl is apparent.

Sperm incorporation into the teleost egg. J. WOLENSKI AND N. J. HART (Rutgers University, New Brunswick, NJ 08854).

During teleost fertilization, a single sperm is incorporated into the egg cytoplasm at a predetermined site of sperm entry (SES). The SES of *Brachydanio*, located in the animal pole, was a circular tuft of 15–20 microvilli that extended into the micropyle of the chorion. Routine TEM of the unactivated egg showed an electron-dense band of "filamentous material" subjacent to the SES. The band was visualized as a meshwork of 60–70 A filaments in cells fixed with glutaraldehyde containing tannic acid and saponin, and postfixed in osmium with $K_3Fe(CN)_6$. The successful fertilizing spermatozoan bound rapidly to the SES. Subsequently, a fertilization cone formed at the SES; its growth resulted in the incorporation of sperm head and midpiece within 1–2 minutes of gamete binding. During sperm entry, the electron-dense band of filaments insinuated itself between the sperm nucleus and the egg plasma membrane. Filaments were difficult to visualize in the core of the fertilization cone. Vesiculation of the sperm nuclear membrane was initiated shortly after sperm entry into the cytoplasm. Eggs pretreated with cytochalasin B (10 μg/ml) bind sperm to the SES. However, sperm were not incorporated into the cytoplasm, suggesting that sperm entry was dependent upon polymerization of actin filaments.

*Sequence analysis of cDNAs encoding mouse protamines 1 and 2 (MP1 and MP2) demonstrates that MP2 is synthesized as a precursor whereas MP1 is not**.* P. C. YELICK, P. A. JOHNSON, K. C. KLEENE, AND N. B. HECHT (Department of Biology, Tufts University, Medford, MA 02155).

Protamines are small, highly basic, sperm-specific proteins that replace histones during the later stages of spermatogenesis, aiding in the condensation and compaction of DNA in the mature sperm nucleus. In mice, there are two protamine variants, cysteine-rich, tryosine-containing MP1, and histidine-rich MP2. A cDNA for MP1 has been identified and sequenced (Kleene *et al.* 1985. *Biochemistry* **24**: 719–722). Here we report the predicted amino acid sequence of a cDNA clone isolated from a testis library that contains a region whose composition closely matches that determined for mature MP2 (Bellvé and Carraway. 1978. *J. Cell Biol.* **79**: 177a). Two independently isolated, overlapping MP2 cDNA clones demonstrate the presence of nucleotide sequence coding for additional amino acid residues 5′ to the region encoding the mature MP2. This data indicates that MP2 is first synthesized as a precursor, in contrast to MP1 whose cDNA sequence codes for the mature protein. MP1 and MP2 cDNAs share an identical 12 nucleotide sequence in the 3′ untranslated region, which may play a role in their coordinte regulation during spermatogenesis.

This work was supported by NIH grant GM29224.

** Best student poster presentation.

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